Exposure of juvenile Atlantic cod (*Gadus morhua*) to water-accommodated fractions of Arabian light crude oil: Biotransformation and DNA damage.

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Master of Science Thesis

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UNIVERSITY OF OSLO, NORWAY
2014

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http://www.duo.uio.no

Print: Reprosentralen, University of Oslo

Acknowledgements

This thesis was written at the Section for Aquatic Biology and Toxicology at the Department of Biosciences, University of Oslo - under the supervision and co-supervision of professor Ketil Hylland and post doc Tor Fredrik Holth, respectively.

First, I would like to thank my supervisors for the opportunity to work with you and learn from two incredibly smart and patient guys. These have been two years packed full of new knowledge for me, and you even squeezed in a little adventure to Iceland – so thanks again!

I want to give a great TAKK FYRIR to my “extra supervisor” dr. Halldór Palmar Halldórsson and to Ásdís Ólafsdóttir for your unlimited kindness, helpfulness and hospitality! From sightseeing-trips to homemade meals - you made my stay in Iceland a true pleasure. Ég vona að við höldum ísambaed!
Thanks also to the Hafrannsóknarstofnun in Grindavík for donating the cod used in this experiment, and Reynir Sveinsson for all his practical help (and fantastic newspaper article).

I need to give my biggest thanks to dr. Vesela Slavcheva Yancheva: for all your help (which was not little..!), your kind heart and last but not least your great company during a month in Sandgerði. I wish you all the best back home in Bulgaria!
I would also like to thank dr. Mazyar Yazdani, Hildegunn Dahl and Lene Fredriksen for all taking time out of their busy schedules to help me in the lab(s).

To my amazing friends at the University: Anne Marie Dalen, June Susanne Berg, Eilen Bjotveit Josefsen (and the rest of “Pensjonistforeninga”), and of course My Hanh Tu. First of all, thank you for your helpful feedback on my writing! But mostly, thank you for making these past 5 years the time of my life! I’ve lived for our looooong lunches, dinner parties and wine-drinking ☺ I dream that we’ll all end up back here, spending our days drinking the horrible coffee in the cafeteria as old, soon-to-be-retired professors. I couldn’t have done this without your support!

To Anine and my mom: I love you both so much. You've been there when I’ve needed you, given me helpful feedback, believed in me and made me believe in myself. Thank you!

Lastly, to my “hubby” Jørgen: You’ve supported me through the ups and downs, you’ve endured all of the craziness, and you’ve been very good at pretending that my ramblings about oil and cod are interesting (though I suspect you’ve been looking forward to me delivering for quite some time now. And that’s ok too ;)). I want you to know that I really, really appreciate it all. I love you with all my heart!

Elena

Abstract

Juvenile Atlantic cod (*Gadus morhua*) were exposed to water-accommodated fractions (WAFs) of Arabian light crude oil in two concentrations for one, two or three weeks. The last group was exposed for three weeks followed by a two-week depuration period in clean water. The experiment was conducted in Sandgerði, Iceland, to test the effects of oil components on biomarkers in organisms living in pristine environments. Selected biomarkers (polycyclic aromatic hydrocarbon (PAH) metabolites in bile, ethoxyresorufin-*O*-deethylase (EROD) activity in gills, hepatic EROD activity, hepatic cytochrome P-450 1A (CYP1A) concentration and comet assay) were tested for differences between the exposed groups and the control group. PAH metabolite measurements indicate that the exposure was successful. Fish exposed to a WAF of crude oil had significant increases in biliary 1-OH-phenanthrene concentrations, EROD activities and CYP1A concentrations compared with control fish – but responses did not appear to be dose-dependent. Levels of CYP1A and EROD activity were highest in the lower exposure group than in the higher exposure group throughout the exposure period. This may have been due to higher amounts of inhibiting substances, or a threshold of maximum induction may have been surpassed by the highest concentration WAF. Differences between groups with regards to these biomarkers were erased after depuration. There was no significant difference between exposed and control fish with regards to 1-OH-pyrene concentrations in bile. Further, the degree of DNA damage was not higher in exposed fish than in control fish. However, there were increases in DNA damage from the levels before exposure was started to levels in exposed and control fish. Damage levels were not bettered after depuration, indicating that two weeks of depuration is not sufficient to mend oil induced DNA damage.

There is a need for more knowledge on oil pollution in pristine environments, and the effects it will have on the organisms inhabiting them – as many of the established biomarkers are customized for use in semi-polluted areas.

This thesis is a part of a larger project called “Pristine Arctic” – a collaboration between universities in Norway, Iceland and Sweden working towards establishing baselines for use in monitoring activities in the Arctic.

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Abbreviations

Abbreviation	Meaning
3-OH-B[a]P	3-OH-benzo(a)pyrene
7-ER	7-ethoxyresorufin
AhR	Aryl hydrocarbon receptor
AhRC	Aryl hydrocarbon receptor complex
AL	Arabian Light
ANOVA	Analysis of variance
AP	Alkylphenol
ARNT	Aryl hydrocarbon receptor nuclear translocator
B[a]P	Benzo(a)pyrene
BSA	Bovine serum albumin
CYP	Cytochrome P-450
CYP1A	Cytochrome P-450 1A
DCM	Dichloromethane
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DTT	DL-Dithiothreitol
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
EROD	7-ethoxyresorufin-O-deetylase
ETC	Emergencies Science And Technology Division (Environment Canada)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-Performance Liquid Chromatography
ICES	International Council for the Exploration of the Sea
ITOPF	International Tanker Owner Pollution Federation Ltd.
NADPH	Nicotineamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NCA	Kystverket (Norwegian Coastal

	Administration)
NIVA	Norsk Institutt for Vannforskning (Norwegian Institute for Water Research)
OLF	Norsk Olje og Gass (Norwegian Oil and Gas Association)
OSPAR	Oslo-Paris Convention (Convention for the Protection of the Marine Environment of the North-East Atlantic)
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate-buffered saline
PW	Produced water
ROS	Reactive oxygen species
RT	Retention Time
TE Buffer	Tris-EDTA Buffer
TMB Plus	Liquid substrate for 3,3',5,5'-tetramethylbenzidine
TTBS	Tris-tween buffered saline
US EPA	United States Environmental Protection Agency
UV	Ultra-Violet
WAF	Water-accommodated fraction
WHO	World Health Organization
WPC	World Petroleum Council

1 Introduction

The ever-increasing demand for oil and gas continuously drive companies to find new production areas. The Arctic region has been found to house large depots of both oil and gas (Gautier et al., 2009; Singh, 2013). The reduction in the summer ice-cover because of environmental changes, and the betterment of oil production technology opens this area for exploration, production and transport (Borgerson, 2008; Gautier et al., 2009; Jonsson et al., 2010; Huebert, 2011; Singh, 2013). With increasing amounts of oil company activity in the Arctic, the potential for discharges, leaks and large-scale spills into this pristine environment will increase dramatically (Abrahamson et al., 2008).

1.1 Crude Oil and Components

Crude oils are complex mixtures of a large number of different substances (Singer et al., 2000), and the specific composition and physicochemical properties will vary in oil from different oil fields (Utvik, 1999; Radović et al., 2012). Crude oil is primarily made up of hydrocarbons, but may also contain metals, nitrogen, sulfur and oxygen (Rossini, 1960). Other substance-groups of interest found in many crude oils are alkylphenols (APs), many of which can have estrogenic properties and the potential to affect endocrine systems in exposed organisms (Martínez-Gómez et al., 2013). Further, naphthenic acids are known to cause sublethal toxic effects (Nero et al., 2006; Knag et al., 2013), as well as causing deformities in fish (Peters et al., 2007). Arabian Light (AL) is a crude oil originating from Saudi-Arabia, and contains approximately 40% aromatics (e.g. polycyclic aromatic hydrocarbons and hetero-polycyclic compounds), 35% saturates (alkanes), 17% resins (ketones, phenols etc.) and 8% asphaltenes (condensed aromatic macromolecules). Compared with other crudes, AL contains high amounts of aromatic substituents and asphaltenes. Most of the toxic effects of oil in marine environments originate from the aromatics, while the asphaltenes are the most persistent components, and provides stability of emulsions of oil in water (Radović et al., 2012). Furthermore, All et al. (1983) showed that AL crude oil contains several toxic trace metals, including cadmium, copper and lead.

The substances in oil that are of most concern in the environment are polycyclic aromatic hydrocarbons (PAHs) (Utvik, 1999; Holth et al., 2008; Grung et al., 2009). PAHs

are a group of molecules that differ in structure and thus function. However, all PAHs are hydrocarbons consisting of two or more fused benzene rings, where some may be alkylated (Hylland, 2006; Holth et al., 2008). Mixtures of PAHs can further contain other substances with known toxic properties, like sulfur and nitrogen-containing heterocyclic compounds (Dizdaroglu et al., 2002). PAHs can originate from both natural and anthropogenic sources: combustion reactions may produce these hydrocarbons (so-called pyrogenic sources); others are produced by living organisms (known as biogenic sources); some result from processes in sediments (termed diagenic sources); and lastly, oil and gas are major sources to PAHs (so-called petrogenic sources) (Neff, 1979, cited in Hylland, 2006). Due to their abilities to cause short- and long-term detrimental effects in aquatic organisms, PAHs in the environment are cause for concern. For this reason, many are included on the priority list of pollutants of both the European Union and the US EPA (Manoli & Samara, 1999). Some PAHs are known to cause oxidative stress (Dizdaroglu et al., 2002), others affect the immune- or endocrine system of the exposed organism (Monteiro et al., 2000; Reynaud & Deschaux, 2006; Hylland, 2006; Arukwe et al., 2008). Geraudie et al. recently (2014) found signs that exposure to PAHs (and APs) can inhibit reproduction. Further, several studies have shown such exposure to impact development (e.g. Rhodes et al., 2005; Mager et al., 2014), and lead to detrimental effects later in life (Huang et al., 2014). Dioxin-like PAHs are able to interact with aryl hydrocarbon receptors (AhR) in cytosol, and consequently lead to dioxin-like toxic effects (Wen et al., 1991; Billiard et al., 2002; Holth et al., 2014). Some PAHs are also recognized as mutagenic and carcinogenic substances (Hendricks et al., 1985; Oh et al., 2012), meaning they may cause mutations and initiate the process of cancer-development. Frequently, this ability is activated through the metabolizing activities of enzymes within the organism (Guengerich & Liebler, 1985; RamaKrishna et al., 1992; Dong et al., 2000).

The introduction of crude oil and oil components into marine environments is a subject of much discussion and concern. The oil production process is a source of significant amounts of pollution into oceans. The drilling of a well creates debris (‘drill cuttings’) that contains oil residue in addition to drilling fluids that are added both to the drill head (‘bit’) and to the drill cuttings themselves to give desired effects (OLF, 2013). Further, introduction of water into the well during extraction of crude oil results in *produced water* (PW) - a water-hydrocarbon mix (Abrahamson et al. 2008), that is the foremost source of pollution from production sites (Hylland et al., 2008; Bakke et al., 2013). Between 2010

and 2012, 93-97% of the total oil pollution into the sea was caused by discharge of PW (OSPAR, 2014). Drain water and displacement water are also regularly discharged from production sites (Bakke et al., 2013). Production sites are allowed certain amounts of discharge into surrounding waters each year, however accidental leaks or large spills may also occur from production sites or during transport (WPC, 2010; Bakke et al., 2013). There were 122 recorded smaller spills (16 m^3 oil in total) in 2012 (Bakke et al., 2013), and there are between 100 and 150 *acute* spills (illegal and sudden) each year along the Norwegian Continental Shelf (NCA, 2013; OLF, 2013).

After oil or oil components enter a body of water, several different weathering processes will take place: spreading by wind and currents; evaporation of volatile, lighter constituents; dispersion from slicks to droplets; emulsion with water; dissolving of constituents that are not completely hydrophobic; biodegradation by microorganisms and photo-oxidation (promoted by UV radiation) (WPC, 2010; ITOPF, 2011; Radović et al., 2012). The weathering or break down of crude oils differ depending on their geochemical origins and refining processes (Radović et al., 2012). Over time, the predominant part of dispersed oil droplets will end up in interaction with sediment particles, sink to the bottom and remain suspended there. Large amounts of oil will at any time be stored in this manner (ITOPF, 2011). Re-release of this oil can take place when water flows through the sediment, and effectively making it bioavailable again (WPC, 2010; ITOPF, 2011; Radović et al., 2012). This sedimentation of oil can create chronic exposure scenarios, by facilitating greater persistence of oil constituents in the environment (Short et al., 2003).

1.2 Biomarkers

WHO (2001) defines a biomarker as “any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease”. In ecotoxicology, the term ‘biomarker’ is used about a measurable departure from normal level of processes at the individual level or lower, as a result of exposure to a specific substance or group of substances (van Gestel & van Brummelen, 1996). Measures of biomarkers in an organism are generally compared with established baseline data, to enable distinctions of actual exposure-responses from individual variations (van der Oost et al., 2003). The use of biomarkers thus make it possible to identify a pollution-threat at an earlier stage than traditional endpoints like reproduction or survival allow (Nahrgang et al., 2010), as these effects higher in the hierarchy (Figure 1.1) are always

preceded by early changes at lower levels in the organism (Bayne et al., 1985). Their use is highly relevant in monitoring activities, where the presence and levels of certain substances are of interest. The establishment of oil production sites in the Arctic Ocean calls for the development of biomarkers that are specialized to monitor organisms adapted to this cold and pristine environment (Abrahamson et al., 2008; Jonsson et al., 2010; Nahrgang et al. 2010).

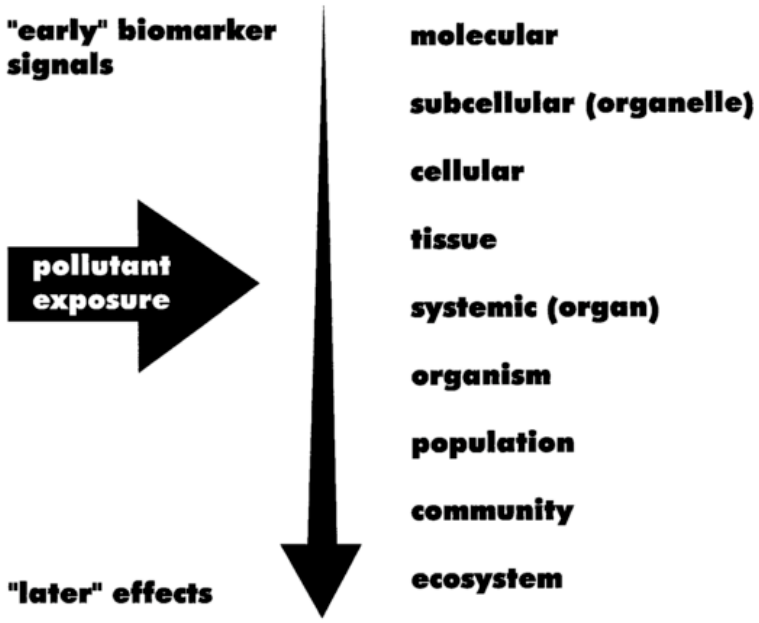

Figure 1.1.Schematic diagram of the successive order of biological responses to pollutant exposure (van der Oost et al., 2003).

How a xenobiotic substance will influence a marine organism is dependent on several factors: Its bioavailability; distribution in the organism; physicochemical properties and ability to interact with receptors etc.; ability to be biotransformed or metabolized; and finally excretion ability in the organism (Bernhoft et al., 1994; Belfroid et al., 1996; Hylland et al., 2009; Martínez-Gómez et al., 2010). Biotransformation is a particularly important factor, as it is a process that either produces more hydrophilic and excretable metabolites, or has the potential of creating detrimentally toxic intermediates (Hahn & Stegeman, 1994). The process is catalyzed by two classes of enzymes: Phase I and phase II. Phase I enzymes catalyze hydrolysis-, oxidation- and reduction-reactions, which through introduction of polar groups leave hydrophobic substances more water-soluble (Behrens & Segner, 2001). Phase II are conjugation reactions that create hydrophilic metabolites by coupling

intermediates from phase I enzymes (or parent substances directly) with internal molecules (Gallagher et al., 1996), generally making them less toxic (Andersson & Förlin, 1992).

Cytochromes P-450 (CYP) is an enzyme-group in a family of heme-containing proteins that play a crucial part in the phase I biotransformation of xenobiotics (Nebert et al., 1991; Bernhardt, 1996; Behrens & Segner, 2001). CYP enzymes originate from a superfamily of genes (the CYP genes) that are found in all organisms (Bernhardt, 1996; 2006). The Cytochrome P-450 1A (CYP1A) isozyme is found in fish (Stegeman, 1989; Goksøyr et al., 1991a; Nebert et al., 1991), mainly focused in the endoplasmic reticulum of microsomes in the liver, but also found in lower densities in other tissues (Guengerich & Liebler, 1985), e.g. the gills of fish. Certain xenobiotic substances are inducers of CYP1A production, such as polychlorinated biphenyls, polychlorinated dibenzofuranes, and PAHs (Andersson & Förlin, 1992). This induction is mediated by the aryl hydrocarbon receptor complexes (AhRC) in the cells of the exposed organism. AhRCs are found in the cytosol of all cells and can bind to planar aryl hydrocarbons, that is, molecules containing at least one aromatic ring (e.g. polycyclic aromatic hydrocarbons (PAHs) [Goksøyr et al., 1991a; Hahn et al., 1993; Whyte et al., 2000]). When a ligand binds to the AhRC in the cytosol, a monomer of the complex - AhR - moves into the cell nucleus and forms a complex with aryl hydrocarbon receptor nuclear translocators (ARNT), and the transcription factor-complex then binds to a promotor region in the CYP1A genes (Hankinson, 1995). This turns the RNA transcription of the CYP1A gene on, increasing the production of CYP1A. This AhR-activation may cause oxidative stress in the exposed cells by inducing of the AhR genes (Cantrell et al., 1996), and the activities of the CYP1A enzymes may also create highly reactive oxygen species (ROS) (Nordblom et al., 1976; Hanukoglu et al., 1993). ROS' can be especially harmful to early life stages and can produce malformed embryos, among other unwanted effects (Cantrell et al., 1996). Further, CYP1A activities may activate the toxicity of certain substances, producing metabolites that are more toxic than the parent substance (Martínez-Gómez et al., 2010). This is the case in the metabolism of the PAH pyrene, where the 1-OH-pyrene metabolite is able to create DNA damage in afflicted cells (Dong et al., 2000; Holth et al., 2014). CYP1A is a membrane-bound enzyme, thus several cellular components as well as DNA are in direct risk of contracting damage from these reactive molecules (Cantrell et al., 1996).

Several biomarkers are based on the inducibility of CYP1A. In cod - as in most vertebrates – PAHs are metabolized relatively efficiently, and thus do not accumulate in

the tissues of the cod. Measuring of PAH metabolites in bile (PAHs main route of elimination) is therefore used as an indicator of exposure to these hydrocarbons (Grung et al., 2009), and as the intermediates and metabolites of PAHs are generally the most toxic forms, measures of PAH metabolites in bile are indirect symptoms of toxic effect in the organism (Aas et al., 2000). After biotransformation by CYP1A in the liver, the metabolites enter the bile canaliculi, flow through the bile duct with the bile and are stored in the gall bladder. From there they may be excreted into the alimentary tract and passed along with faeces following a feeding-event (Aas et al., 1998). As it takes few days (2-3) from exposure to appearance of metabolites in bile of the fish, it is possible to detect exposures relatively early using this method (Ariese et al., 1993; van der Oost et al., 2003). Further, exposure to other substances will not interfere with the measurements of PAH metabolites, as it is a highly specific biomarker (Ariese et al., 1993).

Measurements of ethoxyresorufin-*O*-deethylase (EROD) activity and concentration of CYP1A above background levels can also serve as early warning signals that the organism is being exposed to CYP1A inducing substances. Measurement of EROD activity reflects CYP1A's ability to transform its substrate, 7-ethoxyresorufin (7-ER), into the fluorescent molecule resorufin (Whyte et al., 2000). The enzymatic process involves oxidation of 7-ER, such that the $–CH_2$ group on 7-ER is transformed to –CHOH, which in turn dissociates from the molecule and results in the molecule resorufin (Petrulis et al., 2001). Since the amount of resorufin (the intensity of fluorescence) produced is proportionate to the amount of CYP1A, the measurement of EROD activity proves that receptor-mediated induction of CYP1A has occurred, and thus makes measuring of EROD activity a useful indicative tool (Whyte et al., 2000). The liver is the main tissue in which biotransformation of xenobiotics takes place (Stegeman & Hahn, 1994), explaining why most studies measure EROD activity in hepatic cells (Jönsson et al, 2003). However, as a consequence of their physiology, fish will also be exposed to xenobiotics in large extent through the gills (McKim et al., 1985; Jönsson et al., 2003). Measuring only the hepatic EROD activity may lead to an underestimate of the exposure, as metabolism of CYP1A inducing substances may take place in both gills and other tissues before reaching the liver. Measures of EROD activity in the gill filaments have therefore become more common in recent years (Jönsson et al., 2003). Because of the known ability of CYP1A to produce reactive PAH intermediates, Whyte et al. (2000) argues that EROD activity can be used as a measure of potential DNA damage.

Enzyme-linked immunosorbent assay (ELISA), or indirect ELISA which is the version used in this study, quantifies the relative concentration of CYP1A in the microsomes of a sample (Goksøyr, 1991; Hornbeck, 1991; Butler, 2000). This is accomplished by adding a CYP1A specific antibody to the sample, relying on the fact that antibodies are highly specific in their binding to an antigen (Tijssen, 1985; Hornbeck, 1991). These antibodies are in turn marked with a secondary antibody that has been conjugated with an enzyme (horseradish peroxidase) that can transform an added substrate (TMB Plus) to a measurable color product (Goksøyr, 1991; Hornbeck, 1991). The color intensity produced in the samples is used as a relative measure of the CYP1A concentration in the sample.

Biomarkers based on the inducibility of CYP1A respond rapidly to exposure to low concentrations of pollutants and are relatively compound specific (Goksøyr et al., 1991b). CYP1A induction and EROD activity in an organism are not solely indicators of exposure to substances in their surroundings, however, as they can be affected by other factors - both biotic and abiotic (Andersson & Förlin, 1992; Lyons et al., 2011). It is therefore important, as in any experimental setting, to compare exposed individuals to unexposed control individuals.

Certain substances are known to promote or directly create single- or double-stranded breaks in the DNA molecule (Lee & Steinert, 2003). An example is reactive oxygen species, such as O_2^-, H_2O_2 and •OH, produced during phase I of PAH biotransformation (Guengerish & Liebler, 1985). The comet assay (Singh et al., 1988) can be used to quantify the damage caused by such detrimental substances (Östling & Johanson, 1984; Singh et al., 1988; Eastman & Barry, 1992; Andrade et al., 2004; Hartl et al., 2007). By including a non-exposed control group, one can ensure that the observed effects are due to exposure. Once a sample of cells has been isolated, the cells are embedded in agarose and added to either a microscope slide or specialized sheets of film (Gelbond) (Singh et al. 1988; Nandhakumar et al., 2011). The samples are treated with a lysing solution, so that only the nucleoid is contained within a pocket in the agarose gel (Östling & Johanson, 1984; Singh et al., 1988; Tice et al., 2000). Applying an electric field to these gels will make the free fragments of negatively charged DNA move towards the positive pole of the field (Klaude et al., 1996; Nandhakumar et al., 2011). A largely undamaged DNA molecule is assumed to remain in the pocket, as it encounters a large degree of resistance from the agarose. A relatively damaged DNA molecule, containing several strand breaks and thus

fragments and free ends, will have migration of these free parts while the nested undamaged part of the molecule will be immobile – creating the image of a “comet” when dyed and visualized in a fluorescence microscope (Östling & Johanson, 1984; Singh et al., 1988; Collins et al., 1997; Nandhakumar et al., 2011). The relative amount of DNA damage in each sample is then assessed by comparing the measure of fluorescence in the “tail”, or the migrating fraction of the molecule, with the “head”, or the immobile fraction of the molecule (Cotelle & Férard, 1999; Nandhakumar et al., 2011). Damages to the genetic material is of concern, as it can bring on detrimental biological consequences in cells, organs, the whole organism, or even at population level and in future generations (Lee & Steinert, 2003). As many substances can cause damage, the comet assay is not a specific biomarker. However, it is a useful tool in combination with other biomarkers in natural settings and for use in laboratory experiments.

1.3 Aims of the Experiment

As shown in the previous sections, numerous experiments have investigated the effects of oil (PAH) exposure at various levels and with numerous model organisms. Very few, however, have focused on testing how organisms that have been unexposed to the effects of PAHs before the experiment, respond physiologically to a period of exposure. The present study seeks to contribute to the establishment of baselines for surveillance in pristine regions, like the Arctic, where organisms are thought to be unaffected by pollution from oil and gas production but will be in danger of being exposed when production and transport of oil will inevitably commence there (Abrahamson et al., 2008; Gautier et al., 2009; Singh, 2013). On this notion, the following hypothesis were tested:

H_0 1a: Exposure of Atlantic cod to water-accommodated fractions of Arabian Light crude oil does not increase concentrations of 1-OH-phenanthrene in bile.

H_0 1b: Exposure of Atlantic cod to water-accommodated fractions of Arabian Light crude oil does not increase concentrations of 1-OH-pyrene in bile.

H_0 2: Exposure of Atlantic cod to water-accommodated fractions of Arabian Light crude oil does not increase activity of cytochrome P-450 1A in gills.

H_0 3: Exposure of Atlantic cod to water-accommodated fractions of Arabian Light crude oil does not increase the activity of hepatic cytochrome P-450 1A.

H_0 4: Exposure of Atlantic cod to water-accommodated fractions of Arabian Light crude oil does not increase the concentration of hepatic cytochrome P-450 1A.

H_0 5: Exposure of Atlantic cod to water-accommodated fractions of Arabian Light crude oil does not increase the amount of DNA damage.

2 Materials and Methods

2.1 The System Setup and Experiment

In order to investigate the proposed hypotheses, an exposure experiment was conducted at the Fræðasetrið Nature Center in Sandgerði, Iceland, during November and December 2012. To produce oil water-accommodated fractions (WAFs), seawater was percolated through PVC columns filled with oil-coated gravel as described in Carls et al (1999), modified in Holth et al. (2014) and with the following modifications. The flow-rate was 50 mL/min through peristaltic pumps (WM520S; Watson-Marlow, USA). Water went through Teflon tubes from the column to the tanks containing cod (and blue mussels). All tubings between columns and tanks were in Teflon. To achieve the necessary water flow in the tanks, clean seawater was introduced into the same Teflon tubes at a rate of 250 mL/min. Outlets were fitted at the top of each tank, and the inlets were at the bottom. An overview is presented in Figure 2.1.

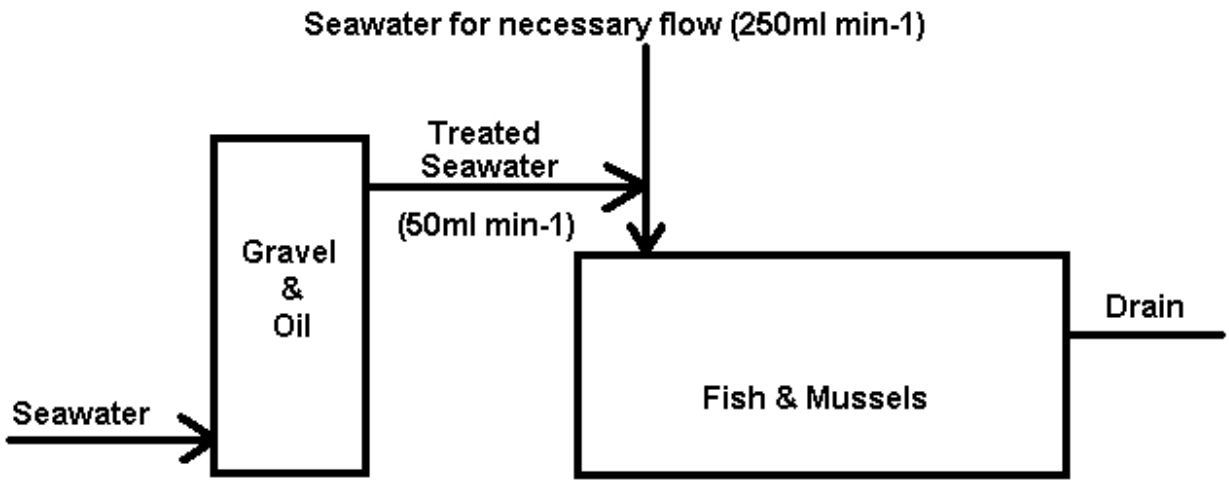

Figure 2.1. The exposure system setup. Clean seawater was percolated through columns filled with oil-coated gravel at a rate of 50 mL/min, with peristaltic pumps. From the columns, the treated seawater (and additional seawater) was pumped into the exposure tanks at a rate of 250 mL/min.

Each tank was covered in black plastic bags, and lids were placed on top of each tank. Salinity, temperature and oxygen-levels were measured daily (see Appendix for details). The tanks were placed into water baths to keep temperatures at 9.5 ± 0.5°C. Seawater was obtained from a fifty meters drill-hole. This seawater is filtered from contaminants as it slowly makes its way through the ground from ocean to reservoir.

2.1.1 Oil Weathering and Gravel Preparation

The oil was weathered artificially by combining one liter of Arabian light (crude oil) with one liter of seawater, then mixed using a magnetic stirrer in a fume hood for 24 hours. The oil/water mix was rested for one hour to separate. During this procedure, the most volatile substances evaporate from the crude oil (Singer et al., 2001). The crude oil was mixed with dichloromethane (2:1 oil:DCM), for even distribution of the oil on the gravel. Three batches of gravel was prepared: 1) “control-gravel” was coated only with the solvent, DCM. 2) “Low dose”-gravel contained 12 g of the weathered crude oil per kilo gravel. 3) “High dose”-gravel contained 36 g of oil per kilo gravel. The gravel was left to air-dry for 24 hours, before filling 1 kg of gravel in each PVC column. The use of oil-coated gravel was thought to imitate a natural exposure scenario, following an oil spill.

2.1.2 Atlantic Cod

The juvenile Atlantic cod used in this experiment were donated by an experimental agriculture station, owned by the Marine Research Institute (Hafrannsóknarstofnun), in Stað in Grindavík. While kept in holding tanks, the cod were fed minced shrimp (1% of cod biomass in tank) daily. The cod were acclimated to the experimental tanks for two weeks before exposure was started. The treatments were randomly assigned to the tanks. After starting exposure, the fish were fed once a week in order to increase the number of gall bladders that contained enough bile to be sampled.

2.2 Sampling

Fish, one per tank, were sampled after one, two and three weeks of exposure. The last group was exposed for three weeks, then left to depurate for two weeks before sampling. The fish were killed by a blow to the head, and blood samples were immediately collected from the caudal vein with 0.6 mm x 30 mm cannulas on 1-mL syringes. The syringes were prewashed with heparin, and filled with an additional drop of heparin and 0.15 mL of PBS with EDTA. Blood samples were kept on ice until further treatment (leukocyte isolation). The fish were measured and weighed, and three gill arches from the left side of each fish were dissected out. Two gill arches were placed – filaments pointing away from each other – in 1 mL HEPES-Cortland buffer in a 24-well plate. The plates were kept on ice, wrapped in aluminum foil until further treatment (measuring EROD activity). The last gill arch from each fish would later be analyzed for histology (not discussed here). The abdomen was

then opened, and liver and gall bladder of each fish were dissected out. The gall bladder was carefully separated from the liver and put in a 0.5 mL tube. The tubes were temporarily kept on ice, then frozen at -20°C. The livers were weighed, and split into replicates and immediately snap-frozen on liquid nitrogen.

2.3 Tissue Preparation

2.3.1 Separation of Blood

In order to isolate the white blood cells, the blood samples were layered on top of density gradients. The gradient consisted of 3 mL of 1.070 g/cm^3 and 2.5 mL of 1.050 g/cm^3 osmoregulated Percoll in 15 mL tubes. The samples were spun for 30 minutes at 2.000 rpm (15°C; WIFUG LABOR Laboratory centrifuge). The white blood cells were then suspended between the two layers with different densities, separated from the rest of the blood. The leukocytes were transferred to a new tube with a Pasteur pipette. The samples were diluted 2x in PBS (with EDTA) and centrifuged for 10 minutes at 2.000 rpm in the centrifuge, to remove the density gradient. The supernatant was removed with a Pasteur pipette, and the pellet was carefully resuspended in 200 µL of PBS (with EDTA).

2.3.2 Isolating Hepatic Microsomes

Samples were thawed on ice, and approximately 1 g of liver sample was weighed out in a homogenization tube with 5 mL homogenizing buffer (phosphate buffer with 0.15 M KCl, 1mM dithiothreitol (DTT) and 5% v/v glycerol). Samples were homogenized while the tubes were submerged in ice, using a motorized Potter-Elvehjem homogenizer at 1.200 rpm. After homogenization, samples were transferred to centrifugation tubes and run in a Multifuge™ 3 S-R centrifuge at 10.000 x *g* for 30 minutes at 4°C. The resulting supernatants (S9 fractions) were transferred into smaller centrifugation tubes and run at 100.000 x *g* for 1 hour at 4°C in a Sorvall™ MTX 150 centrifuge. The supernatants (cytosol) were removed, and the microsomal pellets resuspended in 0.5 mL of ice-cold microsomal buffer (phosphate buffer with 0.15 M KCl and 20% v/v glycerol). With a transfer pipette, the pellets were scraped from the wall of the tubes, mixed with buffer and transferred to 1.5 mL tubes. The suspended pellets were homogenized with a hand-held VWR motorized homogenizer, and each sample divided into three 0.5 mL tubes (one for protein analysis, one for 7-ethoxyresorufin-*O*-deethylase activity analysis (EROD) and one

for enzyme-linked immunosorbent assay [ELISA]) and frozen at -80°C. All work was done on ice (or in cool environments), and the pestle of the homogenizer chilled to avoid degradation of the proteins.

2.4 Analyses

2.4.1 Biliary PAH Metabolites

Preparation of bile samples for HPLC analysis was completed in reduced lighting (based on Krahn et al., 1992 – modified by Grung et al., 2009): a 400 µg/mL stock solution of triphenylamine was made in 80% methanol containing 1% ascorbic acid, and the stock was diluted 25x in 80% methanol with 1% ascorbic acid giving 16 µg/mL triphenylamine internal standard. The bile samples were thawed on ice in the dark. Internal standard, distilled water and β-glucuronidase/aryl sulphatase enzyme (Helix pomata) were kept on ice. A Sartorius BP210S scale was used to weigh samples. Then 20 µL bile, 10 µL of internal standard and 20 µL of enzyme was added to the tube and the weights recorded after each addition. Two standards (mean pyrene-concentrations of 79.3 and 310.5 ng/g bile, in low and high, respectively) were included for every twenty samples. Each sample was then mixed well before they were incubated for 1 hour at 37°C (TS8024; Termaks AS). After incubation, 200 µL of methanol was added to each tube and the tubes were left to cool. The tubes were then centrifuged for 10 minutes at 4000 x *g* using a Multifuge™ 3 S-R centrifuge. The supernatants were transferred to HPLC tubes with transfer pipettes, and the samples were stored at -20°C until further analysis.
The samples were analyzed for PAH metabolites using high-performance liquid chromatography (HPLC) at the Norwegian Institute for Water Research (NIVA) by Dr. Merete Grung. Detections of fluorescence were visualized as chromatograms. Concentrations of metabolites were calculated as the area under the curve of peaks at the known retention times (RT) of each metabolite (Table 2.1).

Table 2.1. Retention times of the measured PAH metabolites. Retention time is given in minutes.

Metabolite	Retention Time (min)
2-OH-naphthalene	6.183
1-OH-phenanthrene	9.663
1-OH-pyrene	12.490
3-OH-benzo(a)pyrene	22.636

Measurements of 2-OH-naphtalene and 3-OH-benzo[a]pyrene were not included in further analysis. There were clusters of peaks around the retention time of 2-OH-naphtalene in all the chromatograms, making quantification impossible (example in Figure 2.2). Only a few samples contained 3-OH-B[a]P, and where it was detected, the curves were pulled sideways at the base (example in Figure 2.3). This indicates wrongful or unreliable detection.

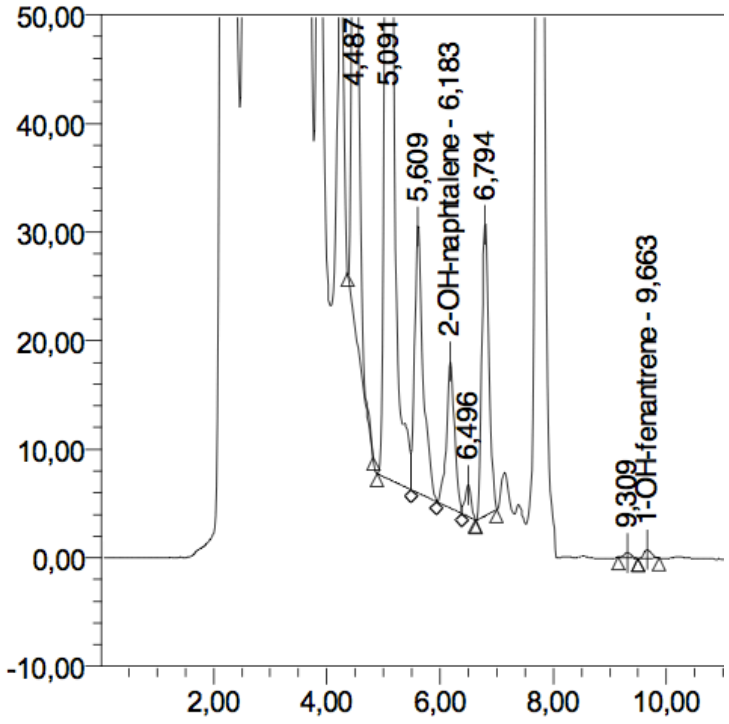

Figure 2.2. Sample chromatogram for peaks around the retention time of 2-OH-naphtalene, making separation difficult. The x-axis depicts retention time, and the y-axis depicts fluorescence. (Sample 52)

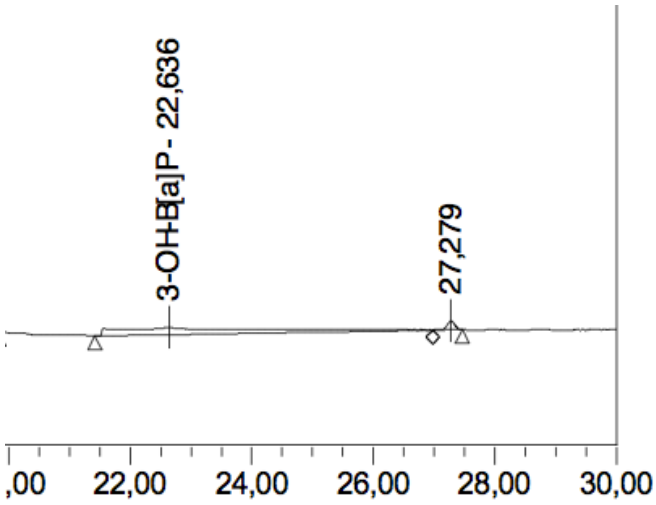

Figure 2.3. Sample chromatogram of a 3-OH-B[a]P measurement that was discarded. The base of the curve has been "pulled" to the right – indicating noise or some other trouble during detection. The x-axis shows the retention time, and the y-axis shows fluorescence. (Sample 52)

2.4.2 Gill 7-Ethoxyresorufin-*O*-Deethylase (EROD) Activity

EROD-buffer was prepared fresh immediately before analysis, mixing 10 mL of HEPES-Cortland buffer (0.4 g KCl, 7.5 g NaCl, 0.25 g $MgSO_4*7H_2O$, 0.25 g $CaCl_2*2H_2O$, 0.35 g $NaH_2PO_4*2H_2O$, 1.45 g HEPES and 1 g glucose in dH_2O up to 1 L) with dicumarol (to 1 µM) and 7-ethoxyresorufin (7-ER) (to 20 µM). Analyses were performed using an indirect light source. The HEPES buffer in the wells was replaced with 500 µL of the EROD buffer. This buffer was replaced after 2 minutes by 500 µL of fresh buffer in order to activate the enzymes. After 30 minutes (1 h for gill arches sampled during the first week, when fish would have lower EROD activity), the buffer was transferred into 1.5 mL tubes. The tubes were covered in aluminum foil and frozen at -20°C until further analysis. The filaments of each gill arch were counted in a binocular microscope.

The frozen tubes were thawed and the content transferred in 200 µL triplicates to a black 96-well microtiter plate. A standard series was made by first preparing a HEPES/PBS solution (14.3 mg of HEPES and 10 mg of glucose in PBS, pH adjusted to 7.7 with NaOH). Resorufin (1 mM) was diluted to 10 µM resorufin in DMSO, and further to 200 nM in PBS/HEPES buffer. This was used as a basis for a 2x dilution series, from 200 nM to 6.25 nM resorufin. PBS/HEPES solution with 0.2% ethoxyresorufin, PBS/HEPES solution with 10% 7-ER and pure PBS/HEPES buffer were used as blanks. Then 200 µL of

the samples, standard series and blanks were added to wells. The plates were read for fluorescence at 535 nm excitation and 585 nm emission, as resorufin is a fluorescent molecule (Dutton et al., 1989).
The mean of the fluorescence from blank wells were subtracted from all other wells, to account for fluorescence in the buffer. A standard curve was created from the fluorescence of the standard series. The mean fluorescence of the triplicate wells was calculated and compared to the standard curve to find the concentration of resorufin in each sample. The concentration of resorufin was divided by the incubation time (60/30 min) and the number of secondary filaments on the gill arches of the fish.

2.4.3 Protein Analysis

Protein concentrations in the microsomal fraction were analyzed according to the method by Lowry et al. (1951), adapted for plate readers. A standard series of bovine serum albumin (BSA) from 1.5 to 0.2 mg/ml in Tris-buffer (12.7 g Trizma HCl and 2.36 g Trisma base in dH_2O up to 1 L) was prepared, and samples were thawed on ice. The samples were diluted 20x in Tris-buffer. Ten µL of samples, blanks (Tris-buffer) and standard series were pipetted into a 96-well plate in triplicates. Twenty-five µL of Bio-Rad DC Protein Assay Reagent A and 200 µL of Bio-Rad DC Protein Assay Reagent B were added to all wells. Plates were carefully swirled, incubated at room temperature for 15 minutes, shaken for 3 seconds and read for absorbance at 750 nm excitation. Samples that returned absorbance readings outside or close to the edges of those from the standard series were diluted either more or less than 20x and tested again. The protein concentration of each processed liver sample was then calculated by constructing a standard curve from the standard series reading, comparing each absorption mean to this curve, accounting for the dilution-degree.

2.4.4 Hepatic 7-Ethoxyresofurin-*O*-Deethylase (EROD) Activity

Hepatic EROD activity was measured using the protocol of Burke and Mayer (1974), adapted for use in plate readers. Frozen aliquots of resorufin, NADPH, 7-etoxyresorufin, microsome samples and reference samples were thawed on ice. A reaction solution was prepared after having diluted the 2.59 mM 7-etoxyresorufin stock in DMSO to a concentration of 0.5 mM. A stock solution of resorufin was prepared by diluting the 1 mM aliquot in potassium phosphate buffer (16.5 g dibasic K_2HPO_4 and 0.7 g monobasic

KH_2PO_4 in 1 L dH_2O) to a concentration of 10 µM. The stock was diluted in the reaction solution in a 2x dilution series from 0.64 µM to 0.01 µM resorufin. A 50 mM NADPH stock solution was diluted in potassium phosphate buffer to 2.4 mM (120 µL of NADPH stock in 2.38 mL of potassium phosphate buffer).
The microsome homogenates were diluted in potassium phosphate buffer to a protein concentration of 1 mg/mL. The diluted samples and reference sample were added to wells of a black 96-well plate in six replicates of 50 µL, the resorufin standard series and reaction solution were added in duplicates of 200 µL, and blank (potassium phosphate buffer) in eight replicates. Ten µL of the 0.32 µM concentration of the standard series was pipetted into three of the replicate wells of liver and reference samples. Two hundred µL of reaction solution and 25 µL of NADPH were added to wells with phosphate buffer and to all wells containing samples and reference samples. The plates were immediately read for fluorescence with 530 nm excitation and 590 nm emission. The plates were read for approximately 8 minutes in 46-second intervals with plate shaking (5 seconds) between readings. The readings were scaled to the highest concentration in the resorufin standard series. All the above steps were completed with only indirect light sources.

The mean fluorescence in the blank wells was subtracted from all wells to account for fluorescence in the buffer. A standard curve was constructed from the standard series, and the mean of each sample's fluorescence was compared to the curve to calculate the concentration of resorufin present. A correction factor was calculated for each plate from readings of the reference samples. To normalize results, the readings of each plate were multiplied with its respective correction factor. Dividing this by the incubation time for the respective plate (seconds) and the amount of protein (mg) in the samples gave EROD activity.

2.4.5 Hepatic Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay on microsomes was completed following the protocol of Goksøyr, 1991 – with modifications according to Sundt et al, 2012.
Samples were kept on ice and diluted in coating buffer (1 capsule of carbonate-bicarbonate in 100 mL dH_2O) to a protein concentration of 10 µg/mL. Mixes of surplus (diluted) samples were used as reference on each plate, refrozen at -80°C before use. One hundred µL of coating buffer was added in four replicates to a clear Nunc Immuno 96-well plate. One hundred µL of the samples and reference samples were added in 4

replicates each, before plated were covered with sealing foil (Nunc BarSeal) and incubated at 4°C overnight.

The plates were washed three times in TTBS buffer (10 L dH_2O with 20 mM Tris-buffer and 5 mL Tween-20) using a ScanWasher 300 plate washer. Three hundred µL of TTBS containing 1% w/w BSA (blocking solution) was added to all wells, before incubating for 60 minutes at room temperature. The plates were then washed three times in TTBS buffer. The primary antibody – Rabbit anti-fish CYP1A (CP226; Biosense Laboratories AS) – was diluted 1000x in antibody buffer (1 L TTBS with 0.1% w/w BSA), and 100 µL was added to all wells having contained samples and reference samples. One hundred µL of antibody buffer was added in four replicates as blanks. The plates were then covered in sealing foil and incubated overnight at 4°C.

All plates were washed three times in TTBS buffer. The secondary antibody – Goat anti-rabbit IgG conjugated with HRP (Horseradish Peroxidase) (GAR-HRP) – was diluted 3000x in antibody buffer, and 100 µL was added to all wells. The plates were then covered with sealing foil, and incubated for 6 hours at 4°C. The plates were all washed five times in TTBS buffer, and 100 µL of coloring buffer (TMB Plus) was added to all wells. The plates were incubated in a dark drawer at room temperature for approximately 15 minutes. The reaction was then stopped by adding 100 µL of 0.18 M H_2SO_4 to all wells, producing a yellow color in the wells. The absorbance was read at 450 nm. Throughout the entire analysis, care was taken to fill all wells from the bottom, without touching the walls or floor of the wells with the pipette tip.

The mean of the absorbance in blank wells was subtracted from all wells. The mean absorbance in the replicate wells were calculated, and divided by the number of seconds the appropriate plate has been incubated with TMB Plus. To normalize for variation between plates (including variation in incubation time), a correction factor was calculated by dividing the mean absorbance of the reference samples of *all* plates with the mean absorbance of the reference sample on each plate. This correction factor was multiplied with the mean absorbance of each sample on the corresponding plate.

2.4.6 Comet Assay on Leukocytes

Agarose for the comet films was prepared daily. Seventy-five mg of agarose was mixed with 10 mL of 10% PBS with 0.5 M EDTA. The agarose was divided in eight 1.5 mL tubes (90 µL per tube), and put on a heat block set to 37°C. Ten µL of the isolated leukocytes

was mixed carefully with the agarose-PBS solution. The agarose-leukocyte solution was mixed well before transferring 10 ul onto a pre-marked Gelbond film lay on a chilled metal plate. When the gels had set (max 10 min), the film was placed in a plastic box filled with lysis buffer (89 mL lysis stock with 1 mL Triton X-100 and 10 mL dH_2O) and were stored at 4°C until further handling. All the above steps were completed in a room with only an indirect light source to minimize risk of additional DNA damage from UV light.

2.4.6-1 Electrophoresis

Unwinding buffer (1.8 L dH_2O with 12 mL concentrated HCl and 200 mL stock solution [240 g NaOH and 7.44 g Na_2EDTA in 2 L dH_2O] [total 2.12 L for four films]) was prepared fresh daily and cooled to 4°C. The films were removed from lysis buffer, rinsed in dH_2O and mounted onto frames to keep them level during electrophoresis. They were then left in unwinding buffer for 40 minutes at 4°C, rinsed in dH_2O and put into the electrophoresis chamber with 1.4 L of unwinding buffer. Voltage in the chamber was measured with a voltmeter (P35761; Cen-Tech Ltd.) to ensure that all films were run under the same conditions. Electrophoresis was run for 25 minutes at approximately 4°C, then rinsed in dH_2O. After rinsing, the films were treated twice with 200 mL of neutralization buffer (96.96 g trizma base in 2 L dH_2O [per 4 films]) for 5 minutes. The films were rinsed in dH_2O, before putting them in 96% rectified ethanol for 5 minutes. Lastly, the films were left in fresh 96% rectified ethanol for 90 minutes, and air-dried overnight.
The electrophoresis was completed at the Norwegian Institute of Public Health.

2.4.6-2 Scoring

The dried films were rehydrated and DNA stained using 40 µL SYBR Gold in 40 µL of TE buffer (20 mL tris-HCl and 2 mL EDTA in ddH_2O up to 1 L) on a rocking platform in a dark room for 20 minutes. Then they were rinsed twice in dH_2O. Ready films were mounted onto Plexiglas plates with a small amount of dH_2O and a cover slide. A Zeiss Scope.A1 fluorescence microscope connected to an AVT Stingray F-046 camera was used for scoring the films. The microscope was set to a blue filter, and the films were scored using the computer program Comet Assay IV (Perceptive Instruments Ltd., UK). A total of 50 cells per gel were scored. The Comet software measures the relative amount of fluorescence in the “head” (the pocket in the agarose where the cell was located) compared to the “tail” (the migrating free strand ends and DNA fragments), and this

relative measure of tail intensity is used as a measure of DNA damage (Collins, 2004). The scoring was completed without direct light exposure.

2.5 Statistical Treatment

The calculations and statistical analyses were done using MS Excel® (Microsoft Corporation) and R version 3.1.0 (The R Foundation for Statistical Computing). Figures were prepared using GraphPad Prism 6 (GraphPad Software, Inc.). The data were assessed for normal distribution and variance equality by Shapiro-Wilk's test of normality (Shapiro & Wilk, 1965) and Levene's test for equality of variances (Levene, 1960), respectively. Where data where not normally distributed and/or variances were unequal, transformations of the data were attempted. If transformations had no effect, the non-parametric Kruskal-Wallis rank test (Kruskal & Wallis, 1952) was chosen to analyze the data. Where data were normally distributed and variances were equal (or transformations were successful), a one-way analysis of variance (ANOVA) (Day & Quinn, 1989) was used to analyze the data.

3 Results

3.1 PAH metabolites in bile

Fish exposed to a WAF of Arabian Light crude oil had significantly higher concentrations of 1-OH-phenanthrene in their bile compared to their respective control groups, when all weeks were compared (Kruskal-Wallis, Table 3.1). Within the separate weeks, the concentrations were significantly higher in exposed fish than in controls in the one-, two- and three-week sampling (Kruskal-Wallis, Table 3.1). The null hypothesis can hence be rejected for these time-points. Measurements are presented in Figure 3.1.

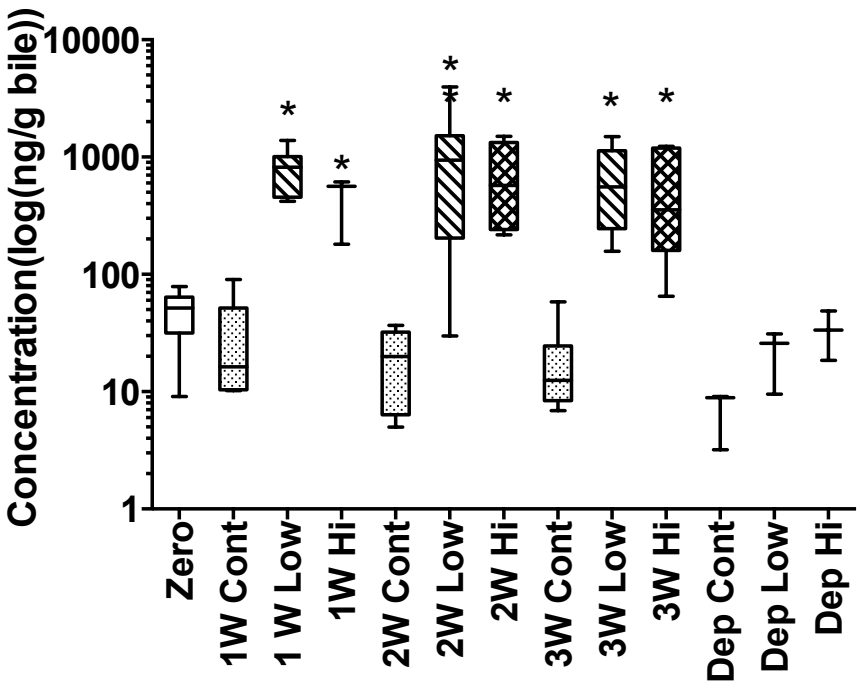

Figure 3.1. 1-OH-phenanthrene (log[ng/g bile]) in cod exposed to AL crude oil WAF in control (Cont), 12 g oil/kg gravel (Low) and 36 g oil/kg gravel (Hi) doses, for one (1W), two (2W), three (3W) or three weeks *plus two weeks depuration* (Dep). Zero indicates measures before exposure. Asterisks indicate significant differences from the corresponding control groups, with an α=0.05.

Fish exposed to a WAF of AL crude oil did not have significantly higher concentrations of 1-OH-pyrene in their bile compared to their respective control groups, when all weeks were compared (one-way ANOVA, Table 3.1). Within the separate weeks, the concentrations were not significantly higher in exposed fish compared to controls in any of

the samplings (one-way ANOVA, Table 3.1). The null hypothesis cannot be rejected. Measurements are presented in Figure 3.2.

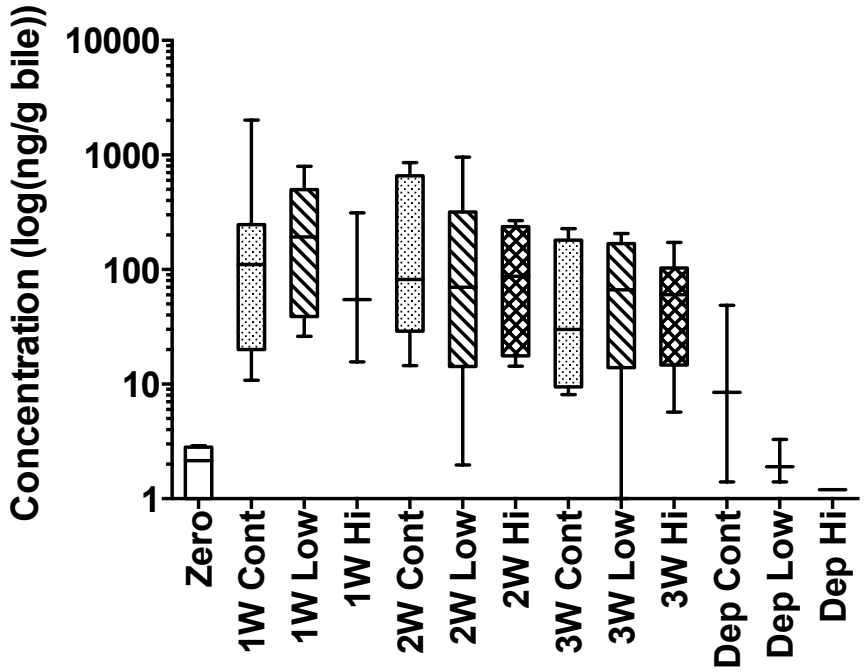

Figure 3.2. 1-OH-pyrene (log[ng/g bile]) in cod exposed to AL crude oil WAF in control (Cont), 12 g oil/kg gravel (Low) and 36 g oil/kg gravel (Hi) doses, for one (1W), two (2W), three (3W) or three weeks *plus two weeks depuration* (Dep). Zero indicates measures before exposure. Asterisks indicate significant differences from the corresponding control groups, with an α=0.05..

Table 3.1. p-values from non-parametric Kruskal-Wallis test (white) and one-way ANOVA (green) on differences in bile concentrations of 1-OH-phenanthrene and 1-OH-pyrene between treated and control groups. Significant differences are indicated with an asterisk (*), when α = 0.05.

Time tested	All times	1 week	2 weeks	3 weeks	Depuration
1-OH-phenanthrene					
p-value	5.7e-09 *	0.0017 *	0.003 *	0.001 *	0.08
Chi-squared	38.0	12.7	11.5	13.8	5.1
df	2	2	2	2	2
1-OH-pyrene					
p-value	0.5	0.8	0.8	0.9	0.4
F-value	0.8	0.2	0.3	0.09	1.1
df	2	2	2	2	2

3.2 Gill Ethoxyresorufin-*O*-Deethylase (EROD) Activity

The EROD activity in gills was significantly higher in fish exposed to WAF of AL crude oil compared with their respective control groups, when all sampling times were compared with each other (Kruskal-Wallis, Table 3.2). When the sampling times were tested separately, there were significantly higher activity levels in exposed fish than in the control group at the one- and three-week sampling (Kruskal-Wallis, Table 3.2). The null hypothesis can hence be rejected for these time-points. Measurements are presented in Figure 3.3.

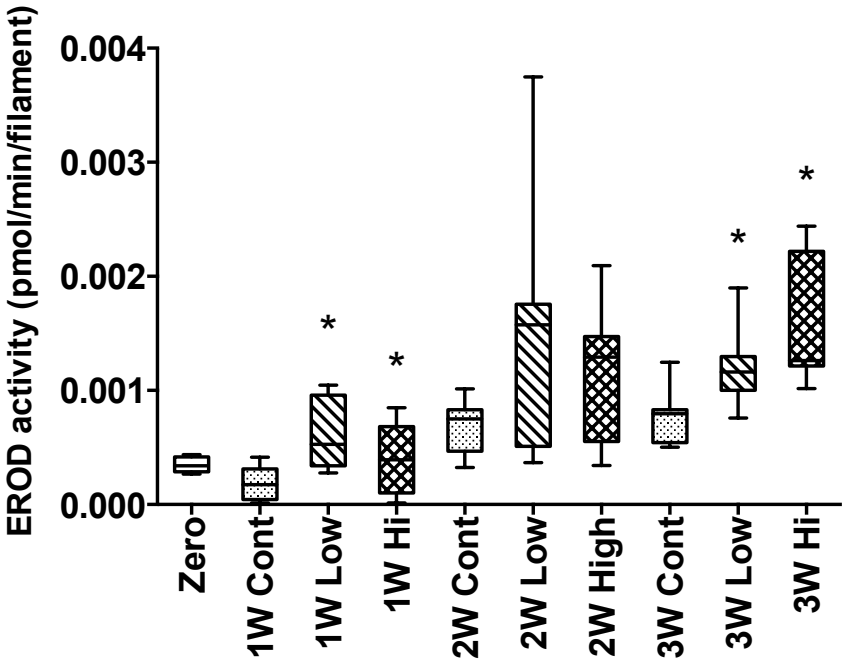

Figure 3.3. EROD activity (pmol resorufin/min/filament) in gills in cod exposed to AL crude oil WAF in control (Cont), 12 g oil/kg gravel (Low) and 36 g oil/kg gravel (Hi) doses, for one (1W), two (2W) or three (3W). Zero indicates measures before exposure. Asterisks indicate significant differences from the corresponding control groups, with an α=0.05..

Table 3.2. p-values from non-parametric Kruskal-Wallis test on differences in EROD activity in gills between treated and control groups. Significant differences are indicated with an asterisk (*), with α=0.05.

Time tested	All times	One week	Two weeks	Three weeks
p-value	0.005*	0.02*	0.2	0.004*
Chi-squared	10.8	8.3	3.8	10.8
df	2	2	2	2

3.3 Hepatic Ethoxyresorufin-*O*-Deethylase (EROD) Activity

Fish exposed to a WAF of AL crude oil had hepatic EROD activity significantly elevated over that of their respective control groups, when all sampling weeks were compared to each other (one-way ANOVA on log-transformed data, Table 3.3). When sampling weeks were tested separately, the EROD activity was significantly higher in exposed fish than in controls at the one, two and three week samplings (one-way ANOVA on log-transformed data, Table 3.3). The null hypothesis can be rejected for these time-points. Measurements are presented in Figure 3.4.

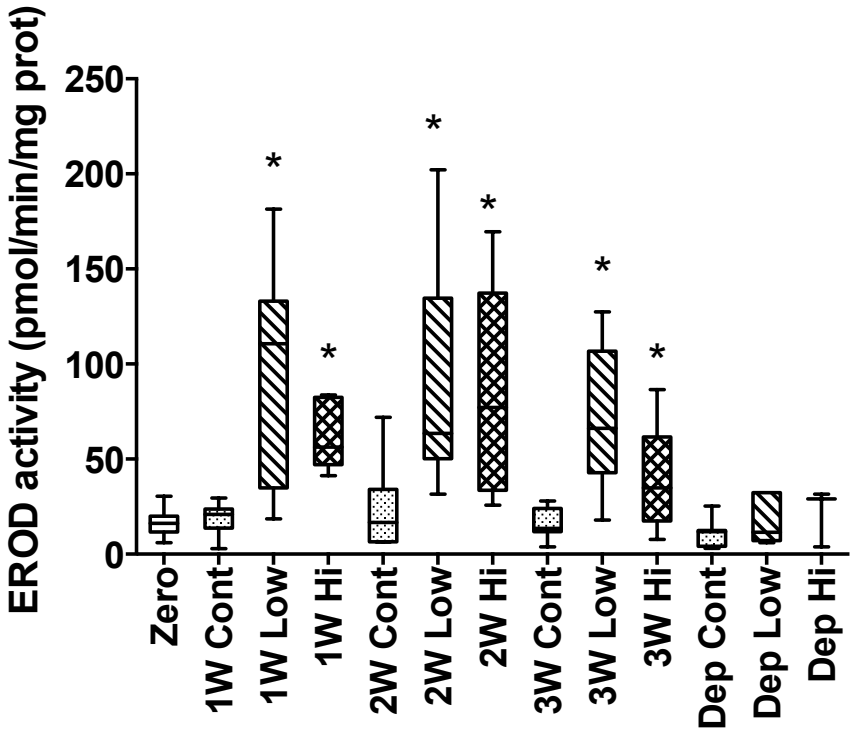

Figure 3.4. Hepatic EROD activity (pmol resorufin/min/mg protein) in cod exposed to AL crude oil WAF in control (Cont), 12 g oil/kg gravel (Low) and 36 g oil/kg gravel (Hi) doses, for one (1W), two (2W), three (3W) or three weeks *plus two weeks depuration* (Dep). Zero indicates measures before exposure. Asterisks indicate significant differences from the corresponding control groups, with an α=0.05.

Table 3.3. p-values from one-way ANOVA on differences in hepatic EROD activity (log-transformed) between treated and control groups. Significant differences are indicated with an asterisk (*), with α=0.05.

Time tested	All times	One week	Two weeks	Three weeks	Depuration
p-value	4.9e-07*	0.001*	0.005*	0.004*	0.6
F-value	18.1	10.3	7.7	7.9	0.5
df	2	2	2	2	2

3.4 Hepatic Enzyme-Linked Immunosorbent Assay (ELISA)

There were significantly higher concentrations of CYP1A induced in the microsomes of fish exposed to WAF of AL crude oil than in their respective control groups, when all weeks were compared with each other (Kruskal-Wallis, Table 3.4). When the weeks were tested separately, the concentrations of CYP1A were significantly higher in exposed fish than in their control group at one-, two- and three-week samplings (Kruskal-Wallis, Table 3.4). The null hypothesis can hence be rejected for these time-points. Measurements are presented in Figure 3.5.

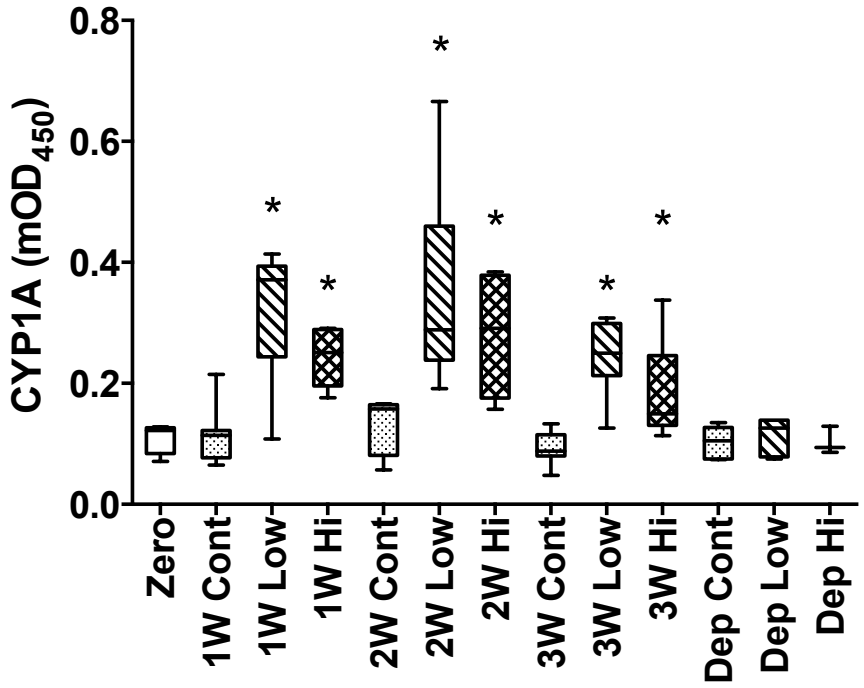

Figure 3.5. Hepatic CYP1A concentrations (mOD_{450}) in cod exposed to AL crude oil WAF in control (Cont), 12 g oil/kg gravel (Low) and 36 g oil/kg gravel (Hi) doses, for one (1W), two (2W), three (3W) or three weeks *plus two weeks depuration* (Dep). Zero indicates measures before exposure. Asterisks indicate significant differences from the corresponding control groups, with an α=0.05.

Table 3.4. p-values from non-parametric Kruskal-Wallis test on differences in CYP1A induction between treated and control groups. Significant differences are indicated with an asterisk (*), with with α=0.05.

Time tested	All times	One week	Two weeks	Three weeks	Depuration
p-value	1.2e-06*	0.008*	0.007*	0.002*	0.8
Chi-squared	27.3	9.6	9.9	12.5	0.5
df	2	2	2	2	2

3.5 Comet Assay on Leucocytes

The median of the tail intensities of the fifty* scored cells was calculated for each gel. The different exposure groups and the exposure times were then compared.

Fish that were exposed to a WAF of AL crude oil had higher degrees of DNA damage than did their respective control groups, when all sampling weeks were compared to each other (Kruskal-Wallis, Table 3.5). When the weeks were tested separately, the exposed fish did

not have higher amounts of DNA damage than their control group in any of the weeks (Kruskal-Wallis, Table 3.5). The null hypothesis cannot be rejected for the separate time-points. Measurements are presented in Figure 3.6.

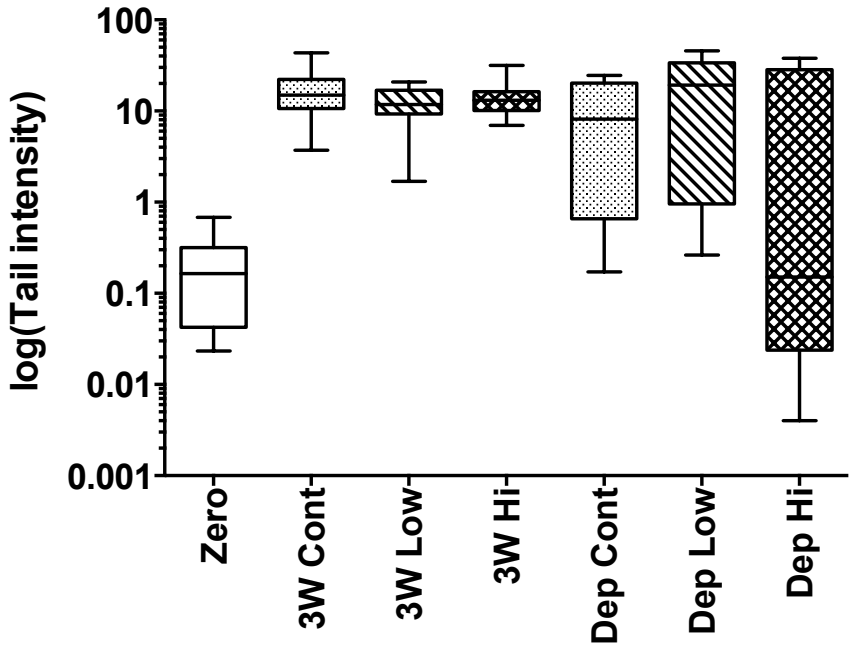

Figure 3.6. DNA damage (log(Tail intensity), Comet Assay IV) in cod exposed to AL crude oil WAF in control (Cont), 12 g oil/kg gravel (Low) and 36 g oil/kg gravel (Hi) doses, for three weeks (3W) or three weeks *plus two weeks depuration* (Dep). Zero indicates measures before exposure. Asterisks indicate significant differences from the corresponding control groups, with an α=0.05.

Table 3.5. p-values from non-parametric Kruskal-Wallis test on differences in DNA damage between treated and control groups. Significant differences are indicated with an asterisk (*), with α=0.05.

Time tested	All times	Three weeks	Depuration
p-value	0.8	0.6	0.2
Chi-squared	0.5	1.1	3.0
df	2	2	2

*not all gels contained enough cells to be able to score fifty.

4 Discussion

4.1 Biliary PAH Metabolites

There were significantly higher concentrations of 1-OH-phenanthrene in the bile of fish exposed to water-accommodated fractions of Arabian light crude oil than in the fish from the control groups at all time points of the exposure period. During the exposure period, exposed fish had a minimum of four times as high concentration as control fish. A change in feeding schedule was implemented after the first week of exposure (from feeding all fish simultaneously, to feeding them seven days before sampling) – likely explaining the concentration-peak at the two-week sampling (Figure 3.1). Starvation has previously been linked to decreasing water-content and increasing concentrations of PAH metabolites in bile (Ariese et al., 1993). There were no significant differences between groups with regards to bile concentrations of 1-OH-pyrene. Figures 3.1 and 3.2 indicate that the concentration of 1-OH-pyrene was generally lower than that of 1-OH-phenantrene in the bile of the cod. This may be due to unequal abilities of the cod to metabolize the two parent molecules, or that other metabolites of the parent substances were present in the bile, other than the ones measured for (Varanasi et al., 1987). However, this may simply have been a result of AL crude oil containing lower concentrations of pyrene than of phenanthrene (ETC, 2000).

The bile concentrations of both PAH metabolites were lower in the fish exposed to the high concentration WAF (36 g oil/kg gravel) than in those exposed to the lower concentration (12 g oil/kg gravel). This seems to indicate the presence of a threshold for maximum EROD activity. Indeed, several studies show that CYP1A induction, and thus EROD activity, is limited by such a threshold (e.g. Rodman et al. 1989; Hahn et al., 1993; Verhallen et al., 1997). As exposure to concentrations of oil above this threshold may increase the time-period in which parent PAHs are retained within the fish, it may also increase the long-term toxicity in the fish. Further, the presence of inhibiting components may contribute to this pattern. One such CYP1A-inhibiting PAH is fluoranthene (Willett et al., 1998), an isomer of pyrene that is found in lower concentrations in AL crude oil (0.37 µg/g oil in 26% weathered crude) (ETC, 2000). Fluoranthene works as an allosteric antagonist on AhR receptors (Willett et al., 1998), meaning that it binds to a site on AhR (not the active site), hindering activation of the receptor, and thus prevents binding of substrates to the active site (Kenakin, 2007). This implies that fluoranthene can prevent

normal metabolism of other PAHs, and the higher concentration of this substance in the high concentration WAF may be the cause of this response pattern. Another hypothesis is that adding 36 g weathered crude per kilo gravel exceeded the amount of oil able to adsorb to the gravel. However, the oil would have been present in the column even if some of it may not have adsorbed to the gravel, thus the WAF would have contained a higher concentration of PAHs than the WAF from the less loaded gravel.

After depuration, there were no differences in PAH metabolite concentrations between groups. As bile is secreted into the intestines following feeding and thus is regularly excreted with faeces, the cod will gradually rid itself of the metabolites contained there (Grung et al., 2009). Thus, keeping the cod in untreated water for two weeks and feeding them regularly, will have made it possible to rid themselves of bodily PAHs and metabolites (Aas et al., 1998). The differences between WAF exposed fish and control groups were thus more or less erased.

The chromatogram of 2-OH-naphthalene contained multiple measurements around the retention time of naphthalene (Figure 2.2), making it difficult to identify the correct peak for quantification (Figure 2.2). Only a small percentage of samples had any measurements of 3-OH-benzo[a]pyrene (3-OH-B[a]P). Those that did all had some degree of curve-base drifting (Figure 2.3) – making the readings unreliable. There results were therefore discarded. AL crude oil is known to contain B[a]P (1.99 µg/g oil in 26 % weathered oil) (ETC, 2000), but several different metabolites may result from the CYP1A-catalyzed metabolism and the levels of 3-OH-B[a]P may have been too low to register reliably.

Some of the cods had little or no bile making any analysis difficult or impossible. The change in feeding schedule increased the ratio of viable bile samples, but upon preparing the bile samples for HPLC analysis more gall bladders were found to be empty. In total, only 71 bladders contained enough bile for analyses. This is a statistical weakness, but does not imply that results lack validity (see Appendix for raw data).

PAH metabolites in the fish bile indicates that exposure has been successful (Grung et al., 2009). From the results it is also clear that, with the exception of the two-week sampling, the concentrations of metabolites decreases over time – in accordance with the static exposure regime in which the oil was not replenished (meaning the WAF concentration decreased). There was, however, not a clear dose-dependent relationship with treatment level for either of the metabolites. The concentrations of 1-OH-phenanthrene in bile during the exposure period varied from 0-1000 ng/g in the low (12 g/kg) group and 0-250 ng/g in the high (36 g/kg) group. Equivalent concentrations for 1-

OH-phenanthrene were from 0-4000 ng/g and 100-1500 ng/g. A baseline-level of 1-OH-pyrene in bile of Atlantic cod was recommended at 4 ng/g bile (Ruus et al., 2003 in Grung et al., 2009). This probably implies that 4 ng/g bile is the limit of metabolites in bile where there are still no observed detrimental effects in the organism. In general, background levels of PAH metabolite concentrations in bile of Atlantic cod range from 0.6-4 ng/g in Norwegian waters (Ruus et al., 2003 in ICES, 2012). Harman et al. (2009) exposed Atlantic cod to a WAF of APs and PAHs in concentrations of 5.4 µg/L (high) and 0.54 µg/L (low), and measured total pyrene metabolites in bile at concentrations of 0-3500 µg/g in high and 0-500 µg/g in low. The concentrations in that study were chosen to correspond to concentrations found in produced water from the North Sea. This is higher than the levels in this thesis, however only 1-hydroxypyrene metabolites were measured here, while Harman and colleagues measures all pyrene metabolites. The concentrations of 1-OH-pyrene measured in this study are also similar to measures in bile of soles (*Solea solea*) exposed to a relatively large (20 000 t) spill from an oil tanker in France (Budzinski et al., 2004). Likely, that implies that the concentrations of WAF chosen for the experiment were realistic in emulating an oil spill in nature.

Ariese and colleagues (1993) mention the possible inaccuracies of comparing PAH metabolite-levels in fish caught in the wild with levels measured in starved individuals, as periods of starvation are linked with concentration of bile constituents. Such a comparison may lead to underestimation of exposure levels, if the feral individuals have fed recently. Base-line establishments should thus be based on individuals which feeding-status closely resembles that of a natural population. Further, the gender of the fish may lead to variability in PAH level, as male and female Atlantic cod have different spawning and feeding behavior (Fordham & Trippel, 1999), and thus their exposure potentials and patterns may differ.

4.2 CYP1A Activity and Concentration

4.2.1 Ethoxyresorufin-*O*-Deethylase (EROD) Activity in Gills

With the exception of the two-week sampling, fish exposed to WAFs of AL crude oil had significantly higher EROD activity in their gill filaments than the control fish, both when all sampling times in the exposure period were tested separately and together. Figure 3.3 clearly shows that there were higher activity levels in both the treated groups than in the

control group at the two-week sampling as well, though the statistical test did not support this. Activity levels were approximately twice as high (or higher) in exposed fish as control fish throughout the exposure period. The median of CYP1A activity at the one- and two-week sampling were higher in the low treatment group than in the high treatment group, i.e. there does not appear to be a clear dose-dependency these weeks. The confounding factors discussed in the previous section, namely CYP1A inhibiting components present in the WAF and limiting of induction above a threshold level, are applicable in this context as well. At the three-week sampling, the activity levels are distributed in a more dose-dependent fashion, possibly indicating that WAF concentrations at this point fell below the maximum induction threshold.

In an exposure study where Atlantic cod was exposed to 1 μM β-naphthoflavone for two days, gill EROD activities were measured to approximately 0.045 pmol/filament/min (Jönsson et al., 2003). Medians in the exposed groups of this study were lower than this (Figure 3.3). As the fish were not exposed to isolated PAHs, but rather a mixture of multiple PAHs (and other oil components), this may result in variable effects on EROD activity. Holth et al. (2014) subjected juvenile Atlantic cod to WAFs of AL crude at 2 and 6 g oil/kg gravel, and measured gill EROD activity means of 0.0032 pmol/filament/min in groups exposed to low concentration WAF and 0.0073 fmol/filament/min in groups exposed to high concentration. This is higher than the means measured in this study, and is worth noting, as the WAF concentrations in Holth's study were approximately one sixth of those used in this study. This might be due to the high number of secondary filaments in the cod gills in the current study. As the filaments on a gill arch are positioned in two parallel rows, it can be difficult to separate the two rows from one another. The filaments on one row were counted and multiplied by two, but errors during counting may have occurred. As there was no evidence of wrongful counting, the results were not altered for this thesis. This may account for the relatively low measured EROD activity here. The cod in the current study were further kept in ultra-clean seawater, which may have contributed to background EROD activity levels lower than in the mentioned study.

4.2.2 Hepatic Ethoxyresorufin-*O*-Deethylase (EROD) Activity and CYP1A Concentration

The hepatic CYP1A concentration and EROD activity in fish exposed to WAFs of AL crude oil were significantly higher than in their respective control groups, when all time-points

were compared with each other. When the weeks were tested separately, both CYP1A concentrations and EROD activities were higher in exposed groups than in control groups at one-, two-, and three-week samplings. Activity levels in exposed fish were at least three times as high as in control fish, and enzyme concentrations at least twice as high. There were no differences between groups after having depurated the fish in clean seawater for two weeks.

EROD measures in gills and liver correspond well with one another in this study. The measured EROD activity and CYP1A concentration in the microsomes follow the same pattern as observed in the gills – there was a clear difference between treatment groups and control, however, the activity levels and CYP1A concentrations in the low treatment group (12 g/kg) were higher than that in the high treatment group (36 g/kg) at all sampling times (Figure 3.4 and 3.5). Thus, not only are the activity levels of the enzymes lower in the high treatment group; there appears to be a lower concentration of enzymes. This indicates that there may be inhibition on CYP1A *production,* not just at the level of enzyme activity. As discussed for EROD activity in gills and measurement of biliary metabolites, this may be due to inhibiting effects of certain PAHs, or the process of CYP1A induction may be limited by a maximum threshold that is surpassed in the high treatment group (Willett et al., 1998). Another possible explanation is that one or more substances in the oil may have been hepatotoxic, thus reducing the normal function of hepatic cells after exposure to the highest concentration, leading to lower induction and enzyme activity. Because of the static exposure design, the concentration of oil components in the water-accommodated fraction will decrease with time. This is mirrored in the overall decrease in activity and protein concentration (Figure 3.4 and 3.5), with the exception of the two-week sampling.

Goksøyr et al. (1991b) showed dose-dependency in Atlantic cod between exposure to a North Sea WAF and CYP1A induction, with induction-levels in the low concentration group between the control group and the high concentration group. However, relatively low concentrations of oil (0.04-0.3 ppm) were used. If the CYP1A induction process is limited above a certain concentration of oil components or level of induction, the doses used in Goksøyr's study may have kept below this threshold. Aas and Klungsøyr (1998) reported EROD activities of between 2 and 25 pmol resorufin/min/mg protein in feral Atlantic cod around three oil fields in Norway. This corresponds well with the control group in this experiment, which kept at a mean of 19.2 pmol resorufin/min/mg protein during the three week exposure period. Further, Sturve et al. (2006) exposed juvenile Atlantic cod to a

dose of North Sea oil corresponding to a water-concentration of 0.5 ppm, and reported EROD activities of 60 pmol resorufin/min/mg protein. This approximates the mean activity in the highest exposure group in the current study (62 pmol/min/mg prot), indicating similar WAF concentrations if the possible influence of seawater purity in the current study is set aside. As mentioned for gill EROD measurements, the lack of pollution in the seawater used in this study may have meant low background levels of induction. Holth et al. (2014) measured mean hepatic EROD in juvenile Atlantic cod to be 12.4 and 20.0 pmol following exposure to 2 g/kg and 6 g/kg AL WAFs, respectively. This corresponds to less than one third of EROD levels measured in this study, and one sixth of the WAF concentrations. This shows that there is not necessarily a 1:1 relationship between concentration and response. Further, Holth et al. (2014) measured hepatic CYP1A concentrations after three weeks of exposure to be at least four times as high in exposed fish as in control fish. This is a greater inter-group difference than in the current study, and may be due to individual variance in CYP1A inducibility. It may also imply that the dose-response curve of CYP1A induction is a process with a steep increase at lower concentrations, and less steep with increasing concentrations. ICES (2012) set a limit of hepatic EROD activity in Atlantic cod to 145 pmol/min/mg protein, under which responses are considered background noise. However, this level was based on cod that were double to triple the length of the cod in this study. As levels in the current study ranged from 10-175 and 25-210 pmol/min/mg in the high and low exposure groups, respectively, this implies that some of the individual measures in the exposed groups would have been dismissed as within background range in a monitoring setting, using these baselines. This stresses the need for specialized baselines for use in pristine areas where background levels of CYP1A inducing substances are thought to be low.

There are numerous factors that can influence the level EROD activity: Due to the known inhibiting properties of estradiol (Hansson & Gustafsson, 1981; Hasselberg et al., 2004), there may be sex-related variability in EROD activity. In rainbow trout (*Oncorhynchus mykiss*), Stegeman and Chevion (1980) found signs that females had lower EROD activity levels than males. Beyer et al. (1996) showed that sex was a major contributor to variance in EROD in Atlantic cod. The cod were caged over sediments of varying pollution-levels, and ran PCA analysis with several markers. Not surprisingly, PAH metabolites in bile was also a large contributor to variance. Further, samplings executed outside of the certain periods connected to spawning (gonad generation and recovery phase) indicated that differences between sexes with regards to CYP1A activity are in tight

relationship with maturity and season (Hylland et al., 2009). The temperature in the surrounding waters is a strong influence on the expression of CYP1A (Lyons et al., 2011). Fish are poikilothermic and thus their body temperature equals that of their surroundings (Förlin et al., 1984). However, Nahrgang et al. (2013) found only statistically insignificant signs of seasonal variation in EROD activity in Atlantic cod. Hasselberg et al. (2004) fed a mixture of APs to Atlantic cod, and measured higher levels of CYP1A expression in females than males. In the same study however, EROD *activity* was lower in exposed groups than controls, indicating inhibiting properties of APs on the CYP1A enzymes. Further, periods of reduced food access may also reduce EROD activity (Wall & Crivello, 1999). As the Atlantic cod in this experiment were juvenile and not yet sexually mature, it was difficult to sex them. However, differences in CYP activity between the sexes do not seem to appear until the fish reach sexual maturity in the (Stegeman & Chevion, 1980). The temperatures in the tanks were kept at a constant level (± 9,5°C), and measured daily. There were no marked spikes or drops in the temperature, salinity nor oxygen-levels in any of the tanks (Figure A1, A-2 and A-3).

Xenobiotic substances can increase or decrease EROD activity in fish, as well as influence how *other* substances affect EROD activity. Combined exposure to substances may yield different effects in an organism than each substance would give separately: Sturve et al. (2006) reported that exposure of Atlantic cod to nonylphenol resulted in a lowering in EROD, as did a combination of North Sea oil and APs. Exposure to the North Sea crude alone gave markedly higher activity levels than in controls (60 pmol/min/mg). As crude oils and their WAFs are intricate mixtures of many different substances, this may make predictions of effects challenging. All of these confounding factors are crucial in the interpretation of the measured EROD activity, as neglecting to do so could result in biased or incorrect conclusions, exposure underestimation (Hylland et al., 2009), and possibly underestimating the need for action.

4.3 Comet Assay (DNA damage)

There were no differences in degree of DNA damage between exposed fish and control fish, neither when all times (three-week and post-depuration sampling) were compared to each other nor when these time points were tested separately. This corresponds well with the results shown in Figure 3.6, where levels of damage in the groups are very similar. However, baseline levels, or the “zero time-sampling”, of damage were significantly lower

(Kruskal-Wallis, $p<0.05$. Not mentioned in results) than in exposed fish, with a mean tail intensity of 0.23. Nevertheless, the null hypothesis could not be rejected. There was no betterment of damage levels in the sampling after a period in clean water, indicating that such damages require longer time to mend, if not irreparable. Other studies have found dose-dependent relationships between PAH exposure and degrees of DNA damage (e.g. Aas et al., 2000; Eidsvold, unpublished; Sanni, pers. comm. in ICES, 2012), and the lack of such a relationship in the current study is not easily fathomed.

The comet gels were stored in lysis buffer for up to 3 months, as electrophoresis was done in Oslo. Contradicting results from long-time storing in lysis buffer have been reported in earlier studies. Nacci et al. (1996) claim that comet gels safely can be stored for months in lysis, while others have shown that the patterns in samples are affected and that the amount of DNA damage in a sample is positively correlated with the storing time in lysis buffer (Belpaeme et al., 1998; Fredriksen, unpublished). This means that this lengthy storing may have raised DNA damage levels above that which resulted from the exposure (and background levels), but do not explain the lack of dose-dependency in damage levels.

Exposure to a number of factors can cause DNA damage: UV-radiation and X-rays are well-known examples, causing single- and double-stranded DNA breaks in exposed cells (Lee & Steinert, 2003; Ciereszko et al., 2005). Cod in this study were kept in plastic-covered tanks throughout the experimental period, but will likely have been exposed to UV in their holding tanks. This is likely not a significant contribution factor, however. Certain PAH are also phototoxic, implying that they become considerably more toxic when exposed to UV (Ankley et al., 1995). 1-OH-pyrene has been linked to DNA strand breaks and adduct-formation in combination with UV radiation (Dong et al., 2000). Thus, exposure to UV would have been of more concern in experimental tanks than in their holding tanks without exposure to PAHs. EROD activity in Atlantic cod was found to be positively correlated with DNA strand breaks in red blood cells (Imrik, unpublished). This is supported by a study on rainbow trout exposed to B[a]P (Curtis et al., 2011). This is, most likely, due to the mentioned intermediates resulting from CYP1A activities, and suggests that a prolonged exposure to CYP1A inducing substances may cause increasing amounts of DNA damage in a fish.

Although PAHs are metabolized efficiently in Atlantic cod, and they do not accumulate in tissues, they may cause harm in the organism. EROD induction is naturally connected to AhR-mediated toxic effects, as CYP1A is a product of the binding of

xenobiotics to the AhR (Whyte et al., 2000). CYP enzymes are capable of producing metabolite-intermediates that can be very reactive (reactive intermediate compounds) (Hahn & Stegeman, 1994), in addition to oxygen species that are known to cause oxidative stress (e.g. Nordblom et al., 1976; Hanukoglu et al., 1993). The main concern regarding PAHs in oceans is their potential for carcinogenesis and mutagenesis. CYP1A is known to take part in the production of a powerful carcinogenic metabolite ([+]7,8-diol-9,10-epoxide) through catalyzing the metabolism of B[a]P (Williams & Buhler, 1984), as well being responsible for bioactivation of B[a]P (Hahn & Stegeman, 1994). There are several other examples of CYP1A-mediated activation of PAHs - that is PAHs becoming more toxic through metabolism catalyzed by CYP1A. The increased degree of toxicity is due to the metabolites' electrophilicity, and leaves them capable of interacting with DNA, proteins and RNA (Holth et al., 2009).

Naturally, DNA strand breaks are not exclusively produced by exposure to PAHs. As crude oil will contain a number of other substances, it is feasible than others will be able to induce breaks in the DNA. Exposure to heavy metals can for instance lead to increased production of lipid peroxidases in fish tissues, and thus increase the level of oxidative stress in the cells (reviewed in van der Oost et al., 2003). This indicates that the metals present in the oil WAF may have contributed to the total amount of DNA damage in this study. Naphthenic acids can have estrogenic effects in fish (Nero et al., 2006; Knag et al., 2013). Gagné et al. (2012) found estrogenic effects to be correlated with oxidative stress, and explain this with their involvement in expression of genes connected to oxidative stress. This may imply contribution of naphthenic acids to the damage level as well.

4.4 The Exposure System and Experimental Design

The salinity in the tanks was kept at a constant level in all tanks throughout the experiment (Figure A-2). The first measure of 49 ppt was most likely not correctly executed, as the level was at a constant ± 31 ppt in all tanks for the remainder of the test period. The temperature fluctuated both between tanks and between days of measurement (Figure A-1) – the lowest measurement being 9.1°C and the highest being 10.1°C. However, the tanks seem synchronous in their peaks and drops in temperature, and there is no apparent location-connected effect on temperature (As tanks 1-12 were placed further from the door, but closer to a window; while tanks 13-24 were closest to the door). The

oxygen-levels in the tanks fluctuated considerably (Figure A-3) – from under 70% in some of the tanks during the first and second week of exposure, to over 90% in the week after depuration. The number of fish (and mussels), unsurprisingly, sunk from sampling to sampling, explaining the rise in oxygen-levels in the last weeks of the experiment. However, the tanks in which the oxygen-levels dipped below 70 % (tanks 14, 15, 16, 17 and 18) were all placed on the upper rack of the same shelf. The oxygen-levels in these tanks seem to have normalized at times, but were relatively consistently lower than in the remaining tanks. This indicates that the circulation in these tanks was not adequate in these particular periods. These (relatively) low levels of oxygen may have had implications for the cod, and may have affected results.

All results obtained from this experiment must be viewed in light of the fact that the WAF would have contained other substance-types than merely PAHs. Even if the most volatile components were separated out during weathering, there would still be hundreds, or even thousands (Singer et al., 2000), of different substances left to affect the cod. If not directly affecting the analysis results reported here, they could have affected other aspects of the fitness of the cod. No further weight will be assigned to this problem, but mentioning of the fact seemed appropriate.

4.5 Higher Effects of Oil Spills in Marine Environments

An oil spill in a marine environment can be harmful for fish and other organisms on several levels. All discussed biomarkers have been found to be associated with more adverse effects in the organism. Induction of CYP1A by hydrocarbons naturally lead to higher metabolic activity in the exposed fish. Chronic increases in metabolic rates will most likely increase the oxygen and energy demand in the fish, and further decrease energy available for other purposes, like growth and reproduction (e.g. Schurmann & Steffensen, 1997). As EROD activity is positively correlated with DNA damage in red blood cells, it may also lead to increasing amounts of damage to the genetic material. PAH-induced DNA damages are particularly worrisome, as they have the potential of influencing not only the individual fish but also future generations (Lee & Steinert, 2003), if the damage for instance occurs in the gametes of the fish. DNA damage in gametes may further impair fertility of the exposed fish. Damages to the genetic material in sperm are irreversible, as no repair mechanisms are present in these cells (Ciereszko et al., 2005). In a larger sense, reduced fertility may cause declines in both populations and species if the oil spill is sufficiently extensive. Increased occurrence of liver damage and neoplasia (tumors of the liver) are also connected to exposure to crude oil (Myers et al., 1998; Aas et al., 2000). Liver lesions and tumors may weaken or hinder normal liver functions, with detrimental results (Myers et al., 1998). The mutagenic and carcinogenic potential of many PAHs (and other oil components) may cause such malfunctions, and potentially shorten the life span of fish. Interference with normal endocrine system functioning (e.g. by APs) can have effects on fish fertility, as well as causing numerous other sublethal effects (e.g. Hasselberg et al., 2004; Sturve et al., 2006; Holth et al., 2011). As eggs, larvae and juvenile stages are the most sensitive to effects from hydrocarbons and other oil components (TemaNord, 2008), entire cohorts may be in risk of reduced functioning or deformities if oil spills occur at or around spawning seasons. Deformities may inhibit swimming abilities, and thus hinder both feeding and anti-predator behaviors. Heintz et al. (2000) have also shown long-term effects on growth rates of fish exposed to crude oil WAFs. Naturally, death before hatching or in early life stages is also possible, and may lead to loss of a cohort, and thus reduction in recruitment that season. The majority of studies on oil spill impacts focus on the effects of the acute phase of the spill. However, the persistence of certain oil components in the environments, such as droplets or sediment-bound residues, implies that there is potential for chronic effects in fish and other organisms (Short et al., 2003).

5 Conclusions

Exposing juvenile Atlantic cod to a water-accommodated fraction of Arabian Light crude oil for up to three weeks resulted in elevated cytochrome P-450 1A induction and EROD activity, and significant increases in 1-OH-phenanthrene concentrations in bile. Null hypotheses H_0 1a, H_0 2, H_0 3 and H_0 4 could hence be rejected. No clear dose-dependent relationship was found for any of these biomarkers; in fact there were higher levels of EROD activity and CYP1A induction in the fish exposed to the lowest concentration of oil than in the fish exposed to the higher concentration. This pattern may have arisen due to the presence of inhibiting substances in AL, or the highest concentration WAF may have caused CYP1A induction to surpass its maximum threshold. A period of depuration erased the differences between groups. There were no significant differences in 1-OH-pyrene concentrations in bile of exposed fish and control fish, thus H_0 1b could not be rejected. This may have been due to AL crude containing lower concentrations of pyrene than phenanthrene, Atlantic cod may be more efficient in metabolism of phenanthrene than pyrene, or there may have been higher levels of other metabolites of pyrene in the bile. There were no differences between groups in the amount of DNA damage, however there were lower levels of damage in fish sampled before exposure was started. Regardless, null hypothesis H_0 5 could not be rejected. Depuration had no apparent effect on the degree of DNA damage in either of the groups. EROD activity measurements in gills and liver showed good levels of agreement, confirming gill EROD as a sensitive and reliable biomarker of exposure to crude oil. Measures of PAH metabolites in bile indicate that the exposure was successful.

Further research

Investigating the apparent concentration threshold in CYP1A induction in juvenile Atlantic cod could be of interest in connection to creating baselines for monitoring and for legislative purposes. As exposure to concentrations that exceed this threshold will leave parts of the total PAH-dose in their parental state, it could potentially lead to higher toxicity within the cod. It could also be a source of underestimation of exposure following a spill, as established biomarkers are based on functioning CYP1A enzymes.

As this study neither compared different temperatures against each other, nor compared typically Arctic species (e.g. Polar cod) to temperate species of fish, the data gathered are not representative of effects following an oil spill in the Arctic. To evaluate the

pertinence of biomarkers established for use in temperate environments in Arctic/pristine environments, such comparative studies may be appropriate. There is without a doubt need for deeper knowledge into how the low temperatures and the possible species differences will affect measurements of biomarkers. Further studies into how organisms acclimated to pristine waters will be affected by exposures to oil spills are also appropriate (and in motion, e.g. the “Pristine Arctic” project of which this thesis was a part).

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Appendix

List of Chemicals

Chemical name	Product number	Producer
β-glucuronidase/aryl sulphatase	1.04114.0002	Merck Millipore
β-NADP reduced tetrasodium salt	N1630	Sigma-Aldrich
Bovine serum albumin	A4503	Sigma-Aldrich
Calcium chloride dihydrate	C3306	Sigma-Aldrich
Carbonate-bicarbonate	C3041	Sigma-Aldrich
D-(+)-Glucose	G8270	Sigma-Aldrich
Dicumarol	M1390	Sigma-Aldrich
Dimethyl sulfoxide	D6416	Sigma-Aldrich
Dipotassium phosphate trihydrate	221317	Sigma-Aldrich
Disodium hydrogenphosphate	S5136	Sigma-Aldrich
DL-Dithiothreitol	D0632	Sigma-Aldrich
EDTA (0.5 M, pH=8.0)	15575-020	Life Technologies
Ethanol (96%)		
Ethylenediaminetetraacetic acid disodium salt dihydrate	15576-028	Life Technologies
Glycerol	G5516	Sigma-Aldrich
Goat anti-rabbit IgG GAR-HRP	A0545	Sigma-Aldrich
Heparin sodium salt	H3393	Sigma-Aldrich
HEPES sodium salt	H7006	Sigma-Aldrich
Hydrogen chloride	K02257817	Merck Millipore
L-ascorbic acid	255564	Sigma-Aldrich
Low Melting Point agarose	16520-050	Life Technologies
Magnesium sulfate heptahydrate	M1880	Sigma-Aldrich
Methanol	32213N	Sigma-Aldrich
Percoll®	17-0891-01	GE Healthcare
Phosphate-buffered saline	D8537	Sigma-Aldrich
Potassium chloride	1.04936.1000	Merck Millipore
Potassium phosphate	P5379	Sigma-Aldrich
Rabbit anti-fish CYP1A antibody	CP226	Biosense Laboratories
Reagent A[1]: alkaline copper tartrate solution	Kit no. 5000-0111	Bio-Rad
Reagent B[1]: diluted Folin reagent	Kit no. 5000-0111	Bio-Rad
Resorufin ethyl ether	E3763	Sigma-Aldrich
Resorufin sodium salt	R3257	Sigma-Aldrich

Sodium chloride	S7653	Sigma-Aldrich
Sodium dihydrogen phosphate	71504	Sigma-Aldrich
Sodium hydroxide	28244.295	VWR Chemicals
Sodium phosphate dibasic (anhydrous)	71640	Sigma-Aldrich
Sodium phosphate monobasic dihydrate	71505	Sigma-Aldrich
Sulfuric acid	1.00731.1000	Merck Millipore
SYBR Gold	S-11494	Life Technologies
TMB Plus	4390A	KEM-EN-TEC Diagnostics
Triphenylamine	T81604	Sigma-Aldrich
Tris hydrochloride	T3253	Sigma-Aldrich
Triton X-100	X100	Sigma-Aldrich
Trizma base	T1503	Sigma-Aldrich
Tween-20	P1379	Sigma-Aldrich

List of Solutions

Preparing bile for HPLC analysis		
Solvent for internal standard		
a) **Dissolved ascorbic acid** (5%)	L-ascorbic acid	0.5 g
	dH_2O	10 mL
b) **Ascorbic acid in methanol**	5% ascorbic acid solution	10 mL
	Methanol	40 mL

Gill EROD activity		
HEPES-Cortland buffer	KCl	0.8 g
	NaCl	15 g
	$MgSO_4*7H_2O$	0.5 g
	$CaCl_2*2H_2O$	0.5 g
	$NaH_2PO_4*2H_2O$	0.7 g
	HEPES	2.9 g
	Glucose	2.0 g
	dH_2O	Up to 2 L
		pH = 7.7
EROD buffer	HEPES-Cortland buffer	35 mL
	Dicumarol (1mM)	35 µL
	Ethoxyresorufin (10mM in DMSO)	70 µL
PBS/HEPES saline solution	PBS solution	1 L
	HEPES	1.43 g

	Glucose	1 g
		pH = 7.7

Liver preparation		
Phosphate buffer (0.1 M)	$NaH_2PO_4*H_2O$	1.173 g/L
	Na_2HPO_4	12.995 g/L
	dH_2O	1 L
		pH = 7.8
Sodium phosphate buffer	Phosphate buffer (0.1 M)	1 L
	KCl (0.15 M in buffer)	11.18 g
Homogenizing buffer	Sodium phosphate buffer	500 mL
	DTT	0.077 g (1mM)
	Glycerol	25 mL (5% v/v)
Microsomal buffer	Sodium phosphate buffer	40 mL
	Glycerol	8 mL (20% v/v)

Protein analysis		
Tris buffer (0.1 M)	Trizma HCL	12.7 g
	Trizma Base	2.36 g
	dH_2O	Up to 1 L
		pH = 8.0

Hepatic EROD activity		
Potassium phosphate buffer (0.1 M)	$K_2HPO_4 * 3H_2O$	21.6 g
	KH_2PO_4 (monobasic)	0.72 g
	dH_2O	1 L
		pH = 8.0
Resorufin ethyl ether solution (0.5 mM)	Resorufin ethyl ether	1 mg
	DMSO	8.29 mL
Resorufin standard (1 mM)	Resorufin sodium salt	11.8 mg
	DMSO	50 mL
β-NADPH solution (50 mM)	β-NADP reduced tetrasodium salt	100 mg
	Potassium phosphate buffer	2.40 mL

ELISA		
Coating buffer (0.005 M)	Carbonate-bicarbonate	1 capsule
	dH_2O	100 mL
TTBS	Trizma HCl (0.02 mole)	31.52 g
	Trizma Base (0.02 mole)	24.23 g

	NaCl	292 g
	dH_2O	Up to 10 L
		pH = 8.5
	Tween-20	5 mL
Blocking solution	TTBS	0.5 L
	BSA	5 g
Antibody buffer	TTBS	1 L
	BSA	1 g

Comet assay		
PBS stock	NaCl	8.5 g
	Na_2HPO_4 anhydrate	0.85 g
	KH_2PO_4	0.54 g
	dH_2O	Up to 100 mL
		pH = 7.4
PBS with EDTA	PBS solution (10% of stock)	98 mL
	EDTA (0.5 M liquid)	2 mL
		pH = 7.4
Lysis stock solution	dH_2O	350 mL
	NaCl	73.05 g
	NaOH	4.00 g
	$Na_2EDTA*2H_2O$	18.61 g
	Trizma base	0.61 g
		pH = 10.0
Lysis buffer	Lysis stock solution	89 mL
	Triton X-100	1 mL
	dH_2O	10 mL
Unwinding/electrophoresis stock solution	NaOH	240 g
	Na_2EDTA	7.44 g
	dH_2O	Up to 2 L
Unwinding/electrophoresis buffer	Unwinding/electrophoresis stock solution	200 mL
	HCl (concentrated)	12 mL
	dH_2O	Up to 2 L
		pH ≈ 13.2
Neutralization buffer	Trizma base	96.96 g
	dH_2O	Up to 2 L
		pH = 7.5
TE buffer	Tris-HCl (0.5 M)	20 mL
	EDTA (0.5 M liquid)	2 mL
	ddH_2O	Up to 1 L
		pH = 8.0
Staining solution (per 2 films)	TE buffer	40 mL
	SYBR Gold (pre-diluted aliquot)	40 µL

List of Lab Equipment

Instrument	Producer
8-Channel Matrix Equalizer Pipette	Thermo Fisher Scientific, Inc.
Analytical scale, BP 210 S	Sartorius, AG
Axio Scope.A1	Carl Zeiss, AG
Digital Multimeter, P35761	Cen-Tech Group, Ltd.
DRI-BLOCK Heater DB2A	Techne, Ltd.
Electrophoresis system	Norwegian Institute of Public Health
Hand-held motorized homogenizer	VWR International, LLC.
Lab centrifuge, 10219	WIFUG, Ltd.
LAB pH meter, PHM 92	Radiometer Analytical, SAS
Laboratory Drying Oven, TS8024	Termaks, AS
Multifuge 3 S-R	Heraceus Holding, GmbH
Peristalic pump, 520S IP31	Watson-Marlow Pumps Group
Potter-Elvehjem motorized homogenizer	Janke & Kunkel GmbH
Standard Orbital Shaker, 3500	VWR International, Inc.
SkanWasher 300	Skatron Instruments, AS
Sorvall MTX 150 Micro-Ultracentrifuge	Thermo Fisher Scientific, Inc.
Stereo Microscope, SZ51	Olympus Co.
Stingray F-046	ALLIED Vision Technologies, GmbH
SynergyMX Platereader	BioTek Instruments, Inc.

Tables and Raw Data

Table A-1: Body measurements of the Atlantic cod used in the exposure study

Code	Weight (gr)	Length (cm)	Liver-weight (gr)
F0-05	20	14	1,72
F0-06	26	15	2
F0-07	20	14,2	1,6
F0-08	19	14	1,65
F0-09	28	15,5	2,71
F0-10	19	13,9	2,53
F0-11	22	14,7	2,25
F0-12	19	14,5	2,28
F0-13	20	14,5	1,7
F0-14	19	14	1,93
F1-01	19	13,8	2,995
F1-02	35	16	3,515
F1-03	24	14,3	2,39
F1-04	20	13,5	1,585
F1-05	18	13,5	1,707

F1-06	31	16,2	2,923
F1-07	20	14	1,67
F1-08	16	13,5	1,352
F1-09	12	12,5	0,97
F1-10	31	16	3,28
F1-11	30	15,5	3,385
F1-12	23	14,5	2,176
F1-13	18	13,5	1,501
F1-14	30	16	2,73
F1-15	24	14,5	3,5
F1-16	25	14,5	2,755
F1-17	19	13,8	1,568
F1-18	16	14	1,482
F1-19	19	13,5	1,501
F1-20	13	11,5	0,485
F1-21	14	12,5	0,216
F1-22	31	15,5	2,925
F1-23	16	13,5	1,127
F1-24	15	12,5	1,082
F2-01	15	12,9	1,231
F2-02	15	13	1,151
F2-03	8	11	0,755
F2-04	8	11	0,92
F2-05	24	13,5	2,224
F2-06	22	14	2,628
F2-07	24	13,5	1,983
F2-08	26	14	1,722
F2-09	22	14	2,573
F2-10	14	12	0,361
F2-11	20	13,5	1,391
F2-12	26	14,5	1,866
F2-13	15	12,5	0,866
F2-14	14	12,5	0,912
F2-15	24	14,5	2,232
F2-16	21	13,5	1,838
F2-17	15	12	1,481
F2-18	10	11	0,509
F2-19	15	12	1,156
F2-20	14	12	0,193
F2-21	47	18	3,282
F2-22	26	15	2,538
F2-23	28	15	2,637
F2-24	13	12	1,077
F3-01	23	14	1,866
F3-02	23	15	2,694

F3-03	35	16,5	3,012
F3-04	36	16	3,577
F3-05	22	13,5	1,666
F3-06	12	11,5	0,886
F3-07	20	12	1,272
F3-08	32	15,5	1,981
F3-09	24	15	2,154
F3-10	19	14	1,781
F3-11	16	13,5	1,227
F3-12	23	14,5	2,815
F3-13	14	13	1,181
F3-14	20	14	2,154
F3-15	10	12	0,718
F3-16	29	15,5	2,932
F3-17	14	12	1,098
F3-18	11	11,5	0,556
F3-19	16	13	1,354
F3-20			
F3-21	31	16	1,711
F3-22	30	15	2,921
F3-23	19	13,5	0,767
F3-24	27	15,5	2,543
F5-01	19,5	13,5	1,48
F5-02	28,6	14,5	1,8
F5-03	19	13,5	1,4
F5-04			
F5-05	20,5	14	1,4
F5-06	14	12	1,15
F5-07	14,7	12	0,75
F5-08	20,6	13,5	1,94
F5-09			
F5-10	28,5	15	2,1
F5-11	19,5	13	1,7
F5-12	28,8	14,5	3,2
F5-13	20,3	13	1,1
F5-14	18,4	13,5	0,9
F5-15	24,4	14	2,1
F5-16	20	14	1,8
F5-17	37	16	2,7
F5-18	37	16	3,2
F5-19	14	12	0,9
F5-20			
F5-21	31	15	2,78
F5-22			
F5-23			

F5-24			

Temperature, Salinity and Oxygen Measurements

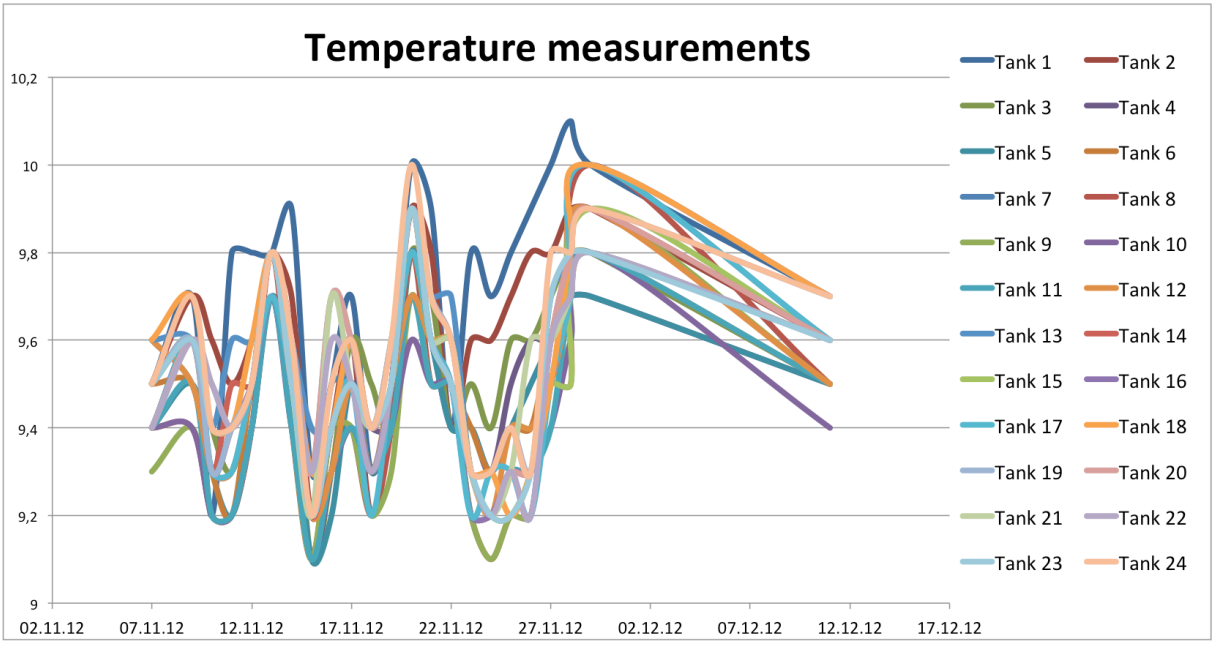

Figure A-1. Temperature was measured in the experimental tanks every day. Each line represents the trend line of one tank throughout the experiment period, measured in °C.

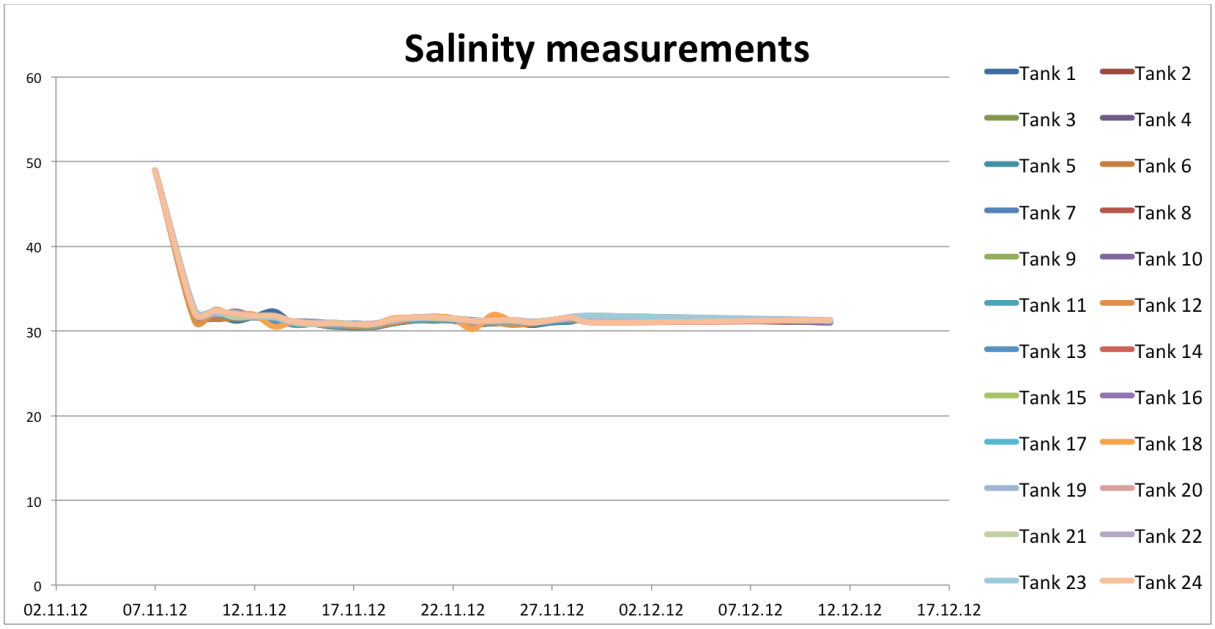

Figure A-2. Measurements of salinity was conducted every day. Each line represents the trend line for one tank throughout the experiment period, measured as parts per thousand (ppt).

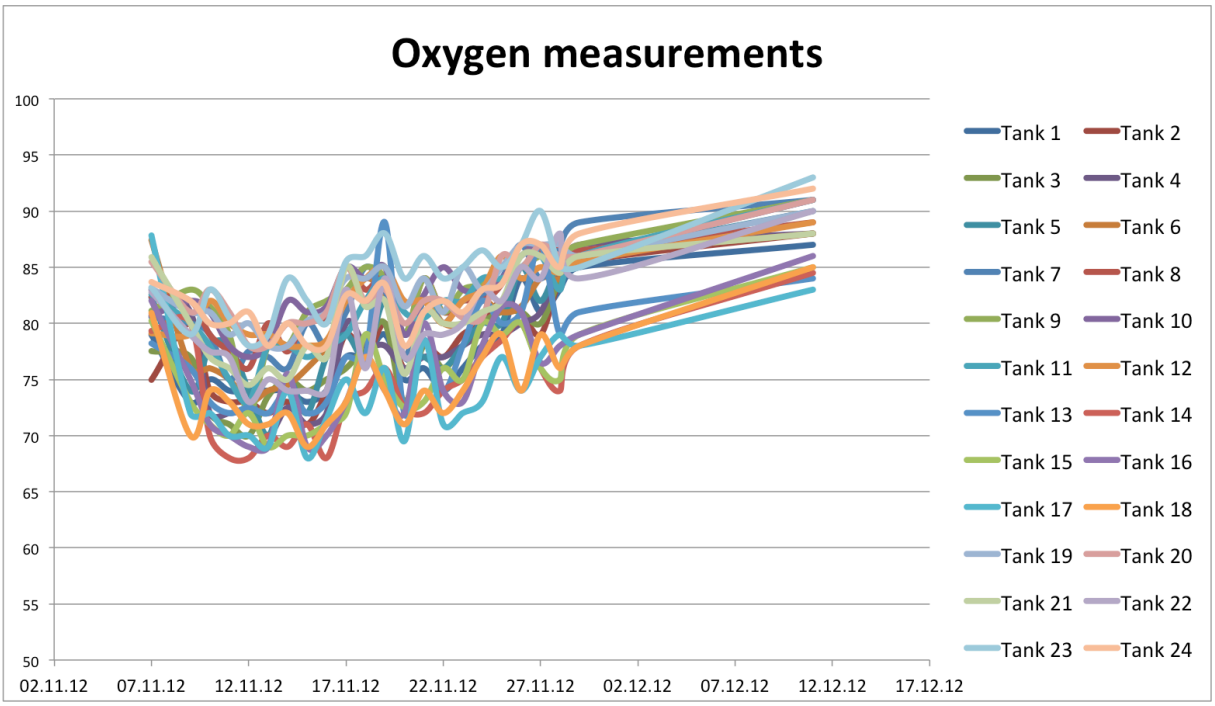

Figure A-3. Oxygen levels were measured in the experimental tanks every day. Each line of the plot represents the trend line of one tank throughout the experiment period, measured in percent (%).

Table A-2: PAH metabolite measurements in bile of the cod, reported in nanograms per gram of bile

Fish code	Assigned treatment	1-OH-phenanthrene concentration (ng/g bile)	1-OH-pyrene concentration (ng/g bile)
F0-01	None		
F0-02	None		
F0-03	None		
F0-04	None		
F0-05	None		
F0-06	None	39.1	2.8
F0-07	None		
F0-08	None		
F0-09	None	50.5	2.4
F0-10	None	9.1	0.7
F0-11	None	59.0	1.9
F0-12	None	52.8	2.9
F0-13	None		
F0-14	None	78.5	0.0
F1-01	12 g/kg	900.1	78.8
F1-02	12 g/kg	1387.0	795.4
F1-03	12 g/kg	424.8	30.9
F1-04	12 g/kg	756.6	306.9
F1-05	36 g/kg	180.3	15.6
F1-06	Control		
F1-07	36 g/kg		

F1-08	Control	90.2	2019.4
F1-09	36 g/kg		
F1-10	Control	10.4	117.2
F1-11	12 g/kg	882.5	62.9
F1-12	Control	18.8	247.2
F1-13	Control	10.2	10.8
F1-14	36 g/kg		
F1-15	12 g/kg	420.5	26.1
F1-16	12 g/kg	547.1	350.2
F1-17	36 g/kg	564.0	54.7
F1-18	36 g/kg		
F1-19	Control	51.5	20.0
F1-20	36 g/kg	614.5	312.8
F1-21	Control	14.1	23.6
F1-22	36 g/kg		
F1-23	Control	16.3	110.7
F1-24	12 g/kg	1042.1	548.9
F2-01	12 g/kg	29.8	2.0
F2-02	12 g/kg	1522.4	318.2
F2-03	12 g/kg	940.3	70.2
F2-04	12 g/kg	3956.1	959.8
F2-05	36 g/kg	216.9	14.3
F2-06	Control	6.5	95.5
F2-07	36 g/kg		
F2-08	Control	36.7	820.8
F2-09	36 g/kg	311.3	27.5
F2-10	Control	6.3	177.3
F2-11	12 g/kg	204.0	14.3
F2-12	Control	32.8	858.7
F2-13	Control	23.4	41.1
F2-14	36 g/kg		
F2-15	12 g/kg	1031.0	66.3
F2-16	12 g/kg	575.3	85.2
F2-17	36 g/kg		
F2-18	36 g/kg	1498.8	146.4
F2-19	Control	16.5	24.9
F2-20	36 g/kg		
F2-21	Control	30.5	68.4
F2-22	36 g/kg	836.0	265.2
F2-23	Control	5.0	14.5
F2-24	12 g/kg		
F3-01	12 g/kg		
F3-02	12 g/kg		
F3-03	12 g/kg	558.2	66.5
F3-04	12 g/kg	157.4	27.9

F3-05	36 g/kg	160.3	14.7
F3-06	Control	16.4	194.8
F3-07	36 g/kg	587.8	60.4
F3-08	Control	8.3	35.7
F3-09	36 g/kg	65.2	5.7
F3-10	Control	27.2	228.1
F3-11	12 g/kg	332.4	0.0
F3-12	Control	58.3	136.8
F3-13	Control	8.7	8.8
F3-14	36 g/kg	160.6	56.2
F3-15	12 g/kg	1493.3	129.8
F3-16	12 g/kg	766.2	206.4
F3-17	36 g/kg	1236.4	103.1
F3-18	36 g/kg	1190.2	172.3
F3-19	Control	6.9	8.1
F3-20	36 g/kg		
F3-21	Control	10.1	11.4
F3-22	36 g/kg	355.3	65.3
F3-23	Control	14.9	24.2
F3-24	12 g/kg		
F5-01	12 g/kg		
F5-02	12 g/kg		
F5-03	12 g/kg	31.0	1.9
F5-04	12 g/kg		
F5-05	36 g/kg	48.4	1.2
F5-06	Control		
F5-07	36 g/kg		
F5-08	Control	8.9	48.8
F5-09	36 g/kg		
F5-10	Control		
F5-11	12 g/kg	25.9	1.4
F5-12	Control	3.2	8.5
F5-13	Control		
F5-14	36 g/kg		
F5-15	12 g/kg		
F5-16	12 g/kg	9.5	3.3
F5-17	36 g/kg	18.4	1.2
F5-18	36 g/kg		
F5-19	Control		
F5-20	36 g/kg		
F5-21	Control	9.1	1.4
F5-22	36 g/kg		
F5-23	Control		
F5-24	12 g/kg		

Table A-3: Analysis results from gill and hepatic EROD, hepatic ELISA and comet assay on leukocytes.

Fish code	Assigned treatment	Gill EROD activity (pmol/min/filament)	Hepatic EROD activity (pmol/min/mg protein)	ELISA/CYP1A concentration (mOD450)	Comet/Tail intensity median
F0-01	None	0.000283	19.0	7.5054E-05	0.33395726
F0-02	None	0.000304	13.6	2.02778E-05	0.271105226
F0-03	None	0.000333	14.7	0.000107298	0.253114151
F0-04	None	0.000429	15.4	7.30742E-05	0.037939496
F0-05	None	0.000345	17.0	1.97222E-05	0.075249416
F0-06	None	0.000438	17.0	0.00011105	0.683866654
F0-07	None	0.000383			0.023318379
F0-08	None	0.000266	11.1	6.2455E-05	0.055483512
F0-09	None		6.0	2.19444E-05	
F0-10	None		9.2	0.000111859	
F0-11	None		21.6	6.62347E-05	
F0-12	None				
F0-13	None		30.5	8.51206E-05	
F0-14	None		20.2	0.000113814	
F1-01	12 g/kg	0.000316	40.4	0.000309651	
F1-02	12 g/kg	0.000277	109.2	0.000360122	
F1-03	12 g/kg	0.000411	112.0	0.000132778	
F1-04	12 g/kg	0.000528			
F1-05	36 g/kg	0.000452	48.9	0.00025162	
F1-06	Control	3.4e-05	2.9	2.83333E-05	
F1-07	36 g/kg	0.000422	49.8	6.38889E-05	
F1-08	Control	0.000256	13.7	0.000101332	
F1-09	36 g/kg	0.000361			
F1-10	Control	0.000329	13.8	5.8311E-05	
F1-11	12 g/kg	0.000525	117.0	0.000373101	
F1-12	Control	0.000239	29.5	0.000187668	
F1-13	Control	0.000415	23.3	0.001625	
F1-14	36 g/kg	0.000848	82.0	0.000324173	
F1-15	12 g/kg	0.001008			
F1-16	12 g/kg	0.000815	18.5	8.73548E-05	
F1-17	36 g/kg	7.6e-05	83.7	0.000258713	
F1-18	36 g/kg	1.4e-05	62.8	0.000168766	
F1-19	Control	6.7e-05	23.8	7.57373E-05	
F1-20	36 g/kg	0.000761			
F1-21	Control	1.4e-05			
F1-22	36 g/kg	0.000175	41.3	0.00022118	
F1-23	Control	0.000108	20.9	5.70554E-05	

F1-24	12 g/kg	0.001046	181.4	0.000400304	
F2-01	12 g/kg	0.000368	202.2	0.00024748	
F2-02	12 g/kg	0.000481	64.2	0.000147588	
F2-03	12 g/kg	0.000596			
F2-04	12 g/kg	0.003749			
F2-05	36 g/kg	0.000354	91.3	8.36111E-05	
F2-06	Control	0.000324	6.4	0.0000525	
F2-07	36 g/kg	0.000342	62.9	0.000156166	
F2-08	Control	0.000405	71.9	8.11111E-05	
F2-09	36 g/kg	0.001150	25.8	0.0001725	
F2-10	Control	0.001015			
F2-11	12 g/kg	0.001745	112.1	0.000411599	
F2-12	Control	0.000699	12.3	3.97222E-05	
F2-13	Control	0.000835			
F2-14	36 g/kg	0.001481	169.6	0.000403779	
F2-15	12 g/kg	0.001473	31.6	0.000608803	
F2-16	12 g/kg	0.001678	56.4	0.000222282	
F2-17	36 g/kg	0.001452	36.2	0.000146829	
F2-18	36 g/kg	0.002095			
F2-19	Control	0.000815	21.4	7.36111E-05	
F2-20	36 g/kg	0.001406			
F2-21	Control	0.000652	21.0	5.0216E-05	
F2-22	36 g/kg	0.001177	126.5	0.000456994	
F2-23	Control	0.000802	6.6	7.81138E-05	
F2-24	12 g/kg	0.001761	62.9	8.52778E-05	
F3-01	12 g/kg	0.001277	66.2	0.000307776	16.9618911
F3-02	12 g/kg	0.001092	18.0	6.47222E-05	1.69781918
F3-03	12 g/kg	0.000758	127.5	0.000269798	9.48189724
F3-04	12 g/kg	0.001125	106.7	0.000228042	9.25552378
F3-05	36 g/kg	0.001214	7.8	0.000015	13.0305758
F3-06	Control	0.000808	17.8	9.36104E-05	3.71115584
F3-07	36 g/kg	0.001016	36.8	0.00012905	6.93732
F3-08	Control	0.000502	28.0	8.42333E-05	14.9888143
F3-09	36 g/kg	0.001262	34.8	0.000137706	16.2613369
F3-10	Control	0.000638	3.8	9.92615E-05	21.1432
F3-11	12 g/kg	0.001303	91.4	0.000252606	16.7704
F3-12	Control	0.000510	24.0	0.000116451	22.6666
F3-13	Control	0.000799	11.9	5.90791E-05	43.3821
F3-14	36 g/kg	0.001235			31.6729
F3-15	12 g/kg	0.001199			13.6050
F3-16	12 g/kg	0.000971	58.0	0.000220509	20.8637
F3-17	36 g/kg	0.002440	27.0	0.000035	15.0957
F3-18	36 g/kg	0.002220			13.1616
F3-19	Control	0.001247	13.6	7.72138E-05	10.305
F3-20	36 g/kg				

F3-21	Control	0.000838	12.1	7.64939E-05	14.9580
F3-22	36 g/kg	0.001553	86.6	0.000270107	10.1255
F3-23	Control	0.000790			11.698
F3-24	12 g/kg	0.001898	42.9	0.000210469	9.895
F5-01	12 g/kg		6.1	6.34231E-05	0.2631
F5-02	12 g/kg				1.654
F5-03	12 g/kg		32.4	6.32261E-05	
F5-04	12 g/kg				
F5-05	36 g/kg				0.2192
F5-06	Control		25.3	8.4227E-05	
F5-07	36 g/kg		29.1	1.69444E-05	
F5-08	Control		12.0	0.000121199	0.254
F5-09	36 g/kg				
F5-10	Control		11.8	0.000113162	8.137
F5-11	12 g/kg		11.5	0.000208297	45.862
F5-12	Control		4.2	9.7185E-05	15.813
F5-13	Control		11.7	0.000347778	24.6254
F5-14	36 g/kg		3.8	0.000114683	37.9031
F5-15	12 g/kg		8.0	2.97222E-05	19.2327
F5-16	12 g/kg		32.4	0.000116622	21.4532
F5-17	36 g/kg		31.5	8.89187E-05	0.08263
F5-18	36 g/kg				0.00443
F5-19	Control		12.5	6.49748E-05	0.17222
F5-20	36 g/kg				
F5-21	Control		3.1	5.65237E-05	1.14497
F5-22	36 g/kg				
F5-23	Control				
F5-24	12 g/kg				

R Script

```
# Metabolites in bile
metabolites<-read.table("Metabolites.txt", header=T, sep=",")
attach(metabolites)

# Check for normal distribution
shapiro.test(metabolites$Conc.Phen)
shapiro.test(metabolites$Conc.Pyr)

# Attempt to log transform to normalize distribution
shapiro.test(log(Conc.Phen))
shapiro.test(log10(Conc.Phen))

# Attempt to put conc in second power
shapiro.test((Conc.Phen)^2)
```

```
# Attempt reciprocal transformation
shapiro.test((Conc.Phen/2))

# Levenes on metabolite-data, to check for variance equality
library(car)
leveneTest(Conc.Phen,Group)
leveneTest(Conc.Pyr,Group)

# Dataset with all times, but excluding zero-time sampling, created
all.times<-metabolites[which(Weeks>0),]

# Datasets of the selected weeks are created
one.week<-metabolites[which(Weeks==1), ]
two.weeks<-metabolites[which(Weeks==2), ]
three.weeks<-metabolites[which(Weeks==3), ]
depuration<-metabolites[which(Weeks==5), ]

# Kruskal-Wallis on all times and the separate sampling times (phenanthrene)
kruskal.test(Conc.Phen~Group, data=all.times)
kruskal.test(Conc.Phen~Group, data=one.week)
kruskal.test(Conc.Phen~Group, data=two.weeks)
kruskal.test(Conc.Phen~Group, data=three.weeks)
kruskal.test(Conc.Phen~Group, data=depuration)

# one-way ANOVA on all times and the separate sampling times (pyrene)
all.times<-lm(Conc.Pyr~Group, data=metabolites)
anova(all.times)
one.weeklm<-lm(Conc.Pyr~Group, data=one.week)
anova(one.weeklm)
two.weekslm<-lm(Conc.Pyr~Group, data=two.weeks)
anova(two.weekslm)
three.weekslm<-lm(Conc.Pyr~Group, data=three.weeks)
anova(three.weekslm)
deplm<-lm(Conc.Pyr~Group, data=depuration)
anova(deplm)

detach(metabolites)

# Gill EROD

gill.erod<-read.table("GillEROD.txt", header=T, sep=",")
attach(gill.erod)

# Check for normal distribution
shapiro.test(gill.erod$EROD.act)

# Attempt to transform
shapiro.test(log(EROD.act))
shapiro.test((EROD.act)^2)
shapiro.test((EROD.act)/2)

# Levene's for variance equality
library(car)
leveneTest(EROD.act,Treatment)

# Dataset with all times, but excluding zero-time sampling, created
all.times<-gill.erod[which(Weeks>0),]

# Kruskal-wallis test on all times
```

```
kruskal.test(EROD.act~Treatment, data=all.times)

# Separating sampling times
zero.time<-gill.erod[which(Weeks==0), ]
one.week<-gill.erod[which(Weeks==1), ]
two.weeks<-gill.erod[which(Weeks==2), ]
three.weeks<-gill.erod[which(Weeks==3), ]

#Kruskal-Walis on the separate times

kruskal.test(EROD.act ~ Treatment, data=one.week)
kruskal.test(EROD.act ~ Treatment, data=two.weeks)
kruskal.test(EROD.act ~ Treatment, data=three.weeks)

detach(gill.erod)

# EROD activity liver

erod_liver<-read.table("EROD liver calc.txt", header=TRUE, sep=",")
attach(erod_liver)

# Check for normal distribution
shapiro.test(EROD_act)

# Levene's test of variance equality
leveneTest(EROD_act, Treatment)

# Attempt to transform
shapiro.test(log(EROD_act))
leveneTest(log(EROD_act), Treatment)

# Dataset with all times, but excluding zero-time sampling, created
all.times<-ero_liver[which(Weeks>0),]

# log-transformation successful.
liver.lm<-lm(log(EROD_act) ~ Treatment, data=all.times)
anova(liver.lm)

# Separating sampling times
one.week<-erod_liver[which(Weeks==1), ]
two.weeks<-erod_liver[which(Weeks==2), ]
three.weeks<-erod_liver[which(Weeks==3), ]
depuration<-erod_liver[which(Weeks==5), ]

# ANOVA on separate times
liver.one<-lm(log(EROD_act) ~ Treatment, data=one.week)
anova(liver.one)
liver.two<-lm(log(EROD_act) ~ Treatment, data=two.weeks)
anova(liver.two)
liver.three<-lm(log(EROD_act) ~ Treatment, data=three.weeks)
anova(liver.three)
liver.dep<-lm(log(EROD_act) ~ Treatment, data=depuration)
anova(liver.dep)

detach(erod_liver)

# ELISA on microsomes

elisa<-read.table("ELISA.txt", header=T, sep=",")
attach(elisa)
```

```
# Test for normal distribution
shapiro.test(elisa$ELISAperSecond)
# Levene's test
library(car)
leveneTest(ELISAperSecond, Group)

# Attempt to transform
shapiro.test(log(ELISAperSecond))
leveneTest(log(ELISAperSecond), Group)
shapiro.test((ELISAperSecond)^2)
leveneTest((ELISAperSecond)^2, Group)
shapiro.test((ELISAperSecond)/2)
leveneTest((ELISAperSecond)/2, Group)

# Dataset with all times, but excluding zero-time sampling, created
all.times<-elisa[which(Weeks>0),]

# Kruskal-wallis test on all times
kruskal.test(ELISAperSecond ~ Group, data=all.times)

#Datasets of the selected weeks are created
one.week<-elisa[which(Days==7), ]
two.weeks<-elisa[which(Days==14), ]
three.weeks<-elisa[which(Days==21), ]
depuration<-elisa[which(Days==35), ]

#Kruskal-wallis on the separate times
kruskal.test(ELISAperSecond ~ Group, data=one.week)
kruskal.test(ELISAperSecond ~ Group, data=two.weeks)
kruskal.test(ELISAperSecond ~ Group, data=three.weeks)
kruskal.test(ELISAperSecond ~ Group, data=depuration)

detach(elisa)

# Comet analysis
comet<-read.table("Comet tail intensity.txt", header=TRUE, sep=",")
attach(comet)

# Test for normal distribution and variance equality
shapiro.test(Tail.int)
leveneTest(Tail.int, Treatment)

# Attempt to transform
shapiro.test((Tail.int)^2)
leveneTest((Tail.int)^2, Treatment)
shapiro.test(log(Tail.int))
leveneTest(log(Tail.int), Treatment)
shapiro.test((Tail.int)/2)
leveneTest((Tail.int)/2, Treatment)

# Dataset with all times, but excluding zero-time sampling, created
all.times<-comet[which(Weeks>0),]

# Kruskal-wallis on all times together
kruskal.test(Tail.int~Treatment, data=all.times)

# Datasets of the selected weeks are created
zero.time<-comet[which(Weeks==0), ]
three.weeks<-comet[which(Weeks==3), ]
```

```
depuration<-comet[which(Weeks==5), ]

# Kruskal-Wallis on different times
kruskal.test(Tail.int ~ Treatment, data=three.weeks)
kruskal.test(Tail.int ~ Treatment, data=depuration)

detach(comet)
```